

Part I

Internal Sample Attenuator Counting (ISAC)

Part II

Analysis of membrane proteins by the use of lipid vesicles

by

Håkan Eriksson



Department of Pure and Applied Biochemistry

Lund 1986

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Let's bring in the coffee
Let's bring in the afternoon
Let's bring in the day to the night

— 1942 —

To my dear parents

Like a bird on the wire
Like a drunk in a midnight choir
I have tried in my way to be free ...

Leonard Cohen

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LIST OF PAPERS

This thesis is based on the following papers, which are referred to by Roman numerals in the text.

- I. H. Eriksson, B. Mattiasson and J.I. Thorell
Use of an internal sample attenuator in radioimmunoassay. Assay of triiodothyronine (T_3) using starch particles containing entrapped charcoal and bismuth oxide in combination with free antibodies
J. Immunol. Methods, 42, 105-113 (1981)
- II. H. Eriksson, B. Mattiasson and J.I. Thorell
Combination of solid-phase second antibody and internal sample attenuator counting techniques. Radioimmunoassay of thyroid-stimulating hormone
J. Immunol. Methods, 72, 117-125 (1984)
- III. H. Eriksson, I. Larsson, J.I. Thorell and B. Mattiasson
Methods of reducing the effect of spontaneous dissociation of antigen-antibody interactions in radioimmunoassays with special reference to internal sample attenuator counting (ISAC)
Scand. J. Clin. Lab. Invest., 43, 575-581 (1983)
- IV. B. Axelsson, H. Eriksson, C. Borrebaeck, B. Mattiasson and H.O. Sjögren
Liposome immune assay (LIA). Use of membrane antigens inserted into labeled lipid vesicles as targets in immune assays
J. Immunol. Methods, 41, 351-363 (1981)
- V. H. Eriksson, B. Mattiasson and H.O. Sjögren
Lectin-mediated binding of liposome-inserted membrane proteins to red blood cells. A method to detect binding of antibodies to purified rat histocompatibility antigen or binding of insulin to the insulin-receptor
J. Immunol. Methods, 75, 167-179 (1984)
- VI. H. Eriksson, B. Baldetorp, B. Mattiasson and H.O. Sjögren
A sensitive method to introduce membrane-bound proteins into recipient cells based on affinity enrichment of lipid vesicles to the recipient cells prior to fusion
Biochim. Biophys. Acta, 815, 417-425 (1985)

This thesis is divided into two sections.

Part I, Internal Sample Attenuator Counting (ISAC), based on papers I - III, is concerned with a general discussion of various aspects of the use of an internal sample attenuator in radioimmunoassays. Papers I and II deal with the development of charcoal and second antibody, based one-step separation techniques in radioimmunoassays, respectively, while paper III concerns a mis-classification problem of the charcoal based separation technique.

Part II, Analysis of membrane proteins by the use of lipid vesicles, based on papers IV - VI, deals with the use of lipid vesicles to investigate the structure and function of integral membrane proteins such as the histocompatibility antigens and the insulin receptor. Papers IV and V are concerned with the detection of membrane proteins incorporated into liposomes, while paper VI describes a method to introduce new membrane proteins into the plasma membrane of viable cells.

INTRODUCTION

Immunoassays are commonly used methods in both analytical and research laboratories. Yalow and Berson were the first to use competition between a labelled ligand and a ligand for a specific antibody in the sample as a way to measure the concentration of that ligand (fig.1). They also developed a method for separation of the unbound ligand from that bound to the antibody and the first radioimmunoassay (RIA), which was for serum insulin, was published in 1959 (Yalow and Berson,1959).

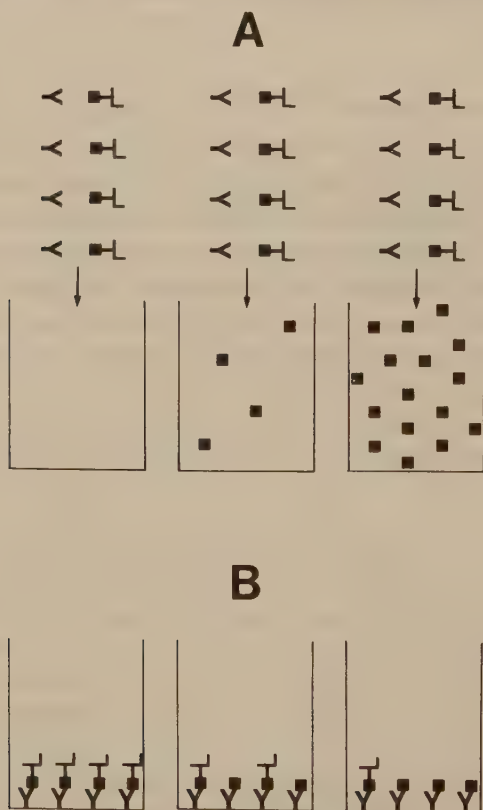


Fig. 1. Schematic presentation of a competitive immunoassay.
A. Incubation of fixed amounts of antibodies (Y), labelled antigen (L) and different concentrations of antigen (■) to be assayed.
B. Counting of the immunologically bound antigen after separation of the bound and free fractions.

Yalow and Berson used a chromatoelectrophoretic separation of the immunologically bound and free ligand. The unbound ligand was retained at the application site on Whatman cellulose paper strips, while the bound fraction migrated away due to a flow of buffer produced by an electric field. Since then many other different methods for the separation of bound and free ligand have been described.

Precipitation techniques which make one of the fractions insoluble, are commonly used methods. The bound fraction can be insolubilized and precipitated by a second antibody directed against immunoglobulins of the species in which the primary antibody was produced (Morgan and Lazarow, 1962, Hales and Randle, 1963) or by chemicals such as ammonium sulphate, ethanol (Chard et al., 1971) and polyethylene glycol (Desbuquois and Aurbach, 1971).

Ligands with a low molecular weight have a strong tendency to adsorb to solid materials and this can be utilized to precipitate the free fraction of the ligand. Some materials with a high adsorptive power that are used for the separation of the free fraction in radioimmunoassays are activated charcoal (Herbert et al., 1965), DEAE cellulose (Frenkel et al., 1966), talcum and silica powder (Rosselin et al., 1966). Instead of separating the ligands in the bound and free fractions after incubation with a primary antibody, the antibody can be added to the assay in an insoluble form. This can be obtained either by covalent coupling of the antibody to a carbohydrate matrix such as Sephadex (Wide and Porath, 1966) or simply by adsorption onto the walls of polystyrene tubes (Catt and Tregear, 1967).

For more than a decade the tracers in the immunoassays were labelled with radioactive isotopes. In 1971 Engvall and Perlmann were the first to use an enzyme, alkaline phosphatase, as a marker in an immunoassay of human IgG. Since then many other enzymes or fluorescent groups have been used as markers in immunoassays (Scharpe et al., 1976, Wisdom, 1976, Aalberse, 1973). The enzymes used in enzyme immunoassay (EIA) can be divided into two groups.

The first group contains enzymes like horse-radish peroxidase, alkaline phosphatase and β -galactosidase which are used in competitive immunoassays with separation of the free and bound fractions of the ligand. These enzymes are also commonly used in sandwich immunoassays (Fig. 2).

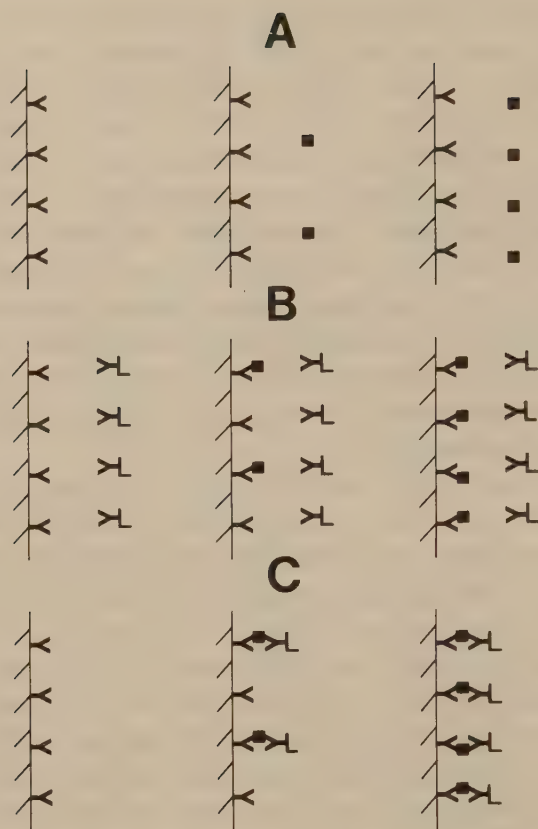


Fig. 2. Schematic presentation of a sandwich immunoassay.

A. Incubation of different concentrations of antigen (■) and antibodies on a solid phase (Y).

B. After washing, incubation with fixed amounts of labelled antibodies (Y-L)

C. Washing and counting.

The second group of enzymes are those whose activity is altered if an antibody binds to a ligand covalently coupled to the enzymes. These include enzymes like lysozyme, glucose-6-phosphate dehydrogenase and malate dehydrogenase. This type of enzyme can be utilized in a competitive assay and no separation between the free and bound fraction of the ligand is required. Since no separation is performed these assays are called homogeneous enzyme immunoassays (Rubenstein et al., 1972, Burd et al., 1978).

The decomposition of the radioactive isotope can not be manipulated in the same way as the activity of an enzyme. However the detection of the decompositions from the free or the bound fraction of the ligand can be manipulated by including an internal sample attenuator in the assay (Thorell,1977, Thorell,1981).

The attenuator can be designed so that the decompositions from either the free or the bound fraction of the ligand can be attenuated (Fig.3).

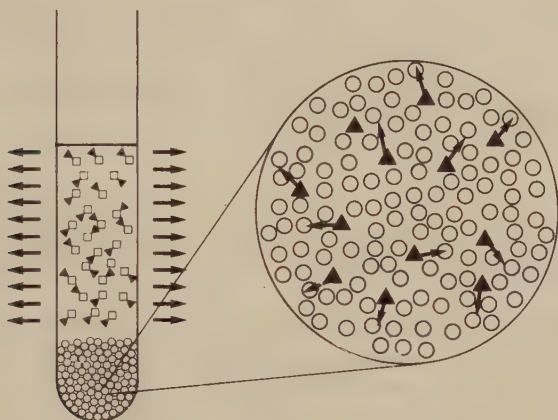


Fig. 3. Schematic presentation of the internal sample attenuator counting, ISAC, technique.

The charcoal adsorber-attenuator reagent is added to an incubation of ^{125}I -labelled antigen ($\blacktriangle$) and a primary antibody ($\square$). After sedimentation of the adsorber-attenuator reagent the immunologically bound fraction of the antigen remains in the supernatant and its gamma radiation ($\longrightarrow$) can be detected. The free fraction of the antigen is adsorbed by the reagent and the gamma radiation from the antigen is attenuated by the co-immobilized bismuth oxide crystals ($\circ$).

Using a second antibody adsorber-attenuator reagent instead, the free fraction of the antigen will remain in the supernatant and the bound fraction of the antigen will be adsorbed and attenuated.

This first part of the thesis, based on papers I - III, is concerned with a general discussion of various aspects of the use of an internal sample attenuator in radioimmunoassays.

SELECTION OF ATTENUATOR

The most commonly used radioactive isotope in radioimmunoassays is ^{125}I . This radionuclide is a pure γ -ray emitter with an energy of 27-35 keV. Due to the low energy of the γ -ray, the radiation penetrates most solid materials rather poorly and in lead the half-thickness of the ^{125}I radiation is as low as 0.015 mm. The absorption of the γ -ray is mainly caused by photoelectric interaction with the K - or L - shell electrons of the attenuator. The highest possible attenuation of the radiation from ^{125}I is obtained if the energy of the attenuator electrons in the K - or L - shells is just below 27 keV. Attenuation characteristics of some of the elements are shown in table I (Thorell, 1981).

TABLE I

Attenuation of 27.4 keV photons by various elements evenly distributed in a 200 μl sphere of the element.

Elements	(Z)	Concentration attenuating 90% of radioactivity g cm^{-3}
Fe	(26)	2.1
Cu	(29)	1.6
Cd	(48)	0.45
I	(53)	2.1
Ba	(56)	1.8
W	(74)	0.62
Hg	(80)	0.53
Pb	(82)	0.60
Bi	(83)	0.54
U	(92)	0.42

Some of the elements listed in table I were immediately discarded due to low attenuation efficiency or to toxicological considerations. In the initial work by Thorell, tungsten powder and different compounds of bismuth were used as attenuators. Finally bismuth oxide, Bi_2O_3 , was chosen as attenuator.

COMBINATION OF ATTENUATION AND ADSORPTION

Two aspects must be considered when introducing an internal sample attenuator into a pellet containing either the immunologically free or the bound fraction in a radioimmunoassay. A schematic presentation of the assay is made in figure 3.

First the amount of attenuator must be sufficient to shield most of the radioactivity from the pellet. However, some leakage of radioactivity from the pellet must be accepted.

Secondly the volume of the attenuator must not be too large. A large pellet causes some of the supernatant to be outside the counting geometry of the gamma counter. In addition, an increased attenuation of the supernatant is obtained with a large pellet.

The easiest way to obtain an attenuating pellet is to add the attenuating material, tungsten or bismuth oxide powder, to the tubes after formation of an immuno-precipitate by a second antibody (Thorell, 1981). However, prior to counting the samples must be centrifuged. Most powders are highly adsorptive and both tungsten and bismuth powder have a tendency to adsorb some of the free fraction and thereby introduce a mis-classification error into the assay.

The difference in density between the immuno precipitate and the attenuator is large. After centrifugation the immuno precipitate is easily found on the top of the pellet, which causes a less effective attenuation.

To avoid the negative aspects and the need for centrifugation, the precipitating and attenuating reagents can be combined by co-immobilization. With this combination the high density of the attenuator circumvents the need of centrifugation. The co-immobilization transforms the separation between free and bound fractions in a radioimmunoassay into a one step procedure, avoiding two of the most laborious steps in radioimmunoassays, the centrifugation and the decantation of the samples.

The first attempts to co-immobilise Bi_2O_3 in an adsorption matrix were made with bismuth oxide crystals with an average diameter of 25 to 50 μm , which made the particles large. The large particles sedimented very rapidly, within a minute. To reduce the sedimentation rate and thereby the need for an extremely high adsorption capacity of the particles, the Bi_2O_3 crystals were ground to microparticles with a diameter less than 5 μm . The decrease of the size of the crystal made it possible to obtain particles with a slower sedimentation rate. The particles also became more densely packed with Bi_2O_3 .

Block-polymerization

Bismuth oxide and activated charcoal can be entrapped in an agarose matrix by block-polymerization. The gel is then disintergrated to obtain particles which possess both adsorption properties to low molecular weight ligands and ^{125}I -attenuating properties (Thorell, 1981 and Paper III). The adsorber-attenuator reagent can be utilized in radioimmunoassays to adsorb and attenuate the free fraction of the ligand.

Bead-polymerization

The block-polymerization procedure results in particles with a wide size distribution. A more narrow distribution in particle size is obtained by bead-polymerization.

Initially bead-polymerization with agarose according to previously published procedures was tried (Hjerten, 1962). The beads, however, became very large and due to the entrapment of Bi_2O_3 they sedimented within a minute. To obtain smaller spheres, starch was used as a matrix (Mosbach and Schröder, 1979). Entrapment of charcoal in the starch matrix reduced the adsorption capacity. After entrapment in the spheres, a suspension containing 17 mg of entrapped charcoal had the same adsorption capacity as 6 mg of dextran coated charcoal. As expected the attenuation of the γ -radiation from the adsorbed tracer increased with increasing amounts of entrapped Bi_2O_3 . However, the leakage from the pellet could not be reduced below 15 % of the adsorbed activity (Paper I).

In general this adsorber-attenuator reagent can be used instead of dextran coated charcoal in all competitive radioimmunoassays using ^{125}I as a tracer. The reagent has been used in radioimmunoassays of thyroid hormones (Paper I), gastrin (Andersen and Jaffe, 1984), steroid hormones and digoxin (Eriksson unpublished). The precision, reproducibility and correlation to other methods are exemplified with a radioimmunoassay of triiodothyronine (T_3) in Paper I.

To obtain a second antibody attenuator reagent, goat anti rabbit-IgG was coupled by the CNBr method (Axén et al., 1967) to starch spheres containing entrapped bismuth oxide. The starch spheres showed a low binding capacity for rabbit IgG. Instead small agarose spheres were used as a second antibody attenuator reagent (Paper II).

The agarose spheres had a high total binding capacity for rabbit IgG. However only a small amount of the binding capacity (1 - 2 %) was used when the binding had to take place during the sedimentation of the spheres (fig. 4).

The second antibody adsorber-attenuator reagent has been used in radioimmunoassays with dilutions of the primary antibody ranging from 1:2500 (thyroxine, T_4) to 1:225.000 (progesterone). The precision, reproducibility and correlation to other methods are exemplified with a radioimmunoassay of thyroid-stimulating hormone (TSH) in Paper II.

Precipitable activity %

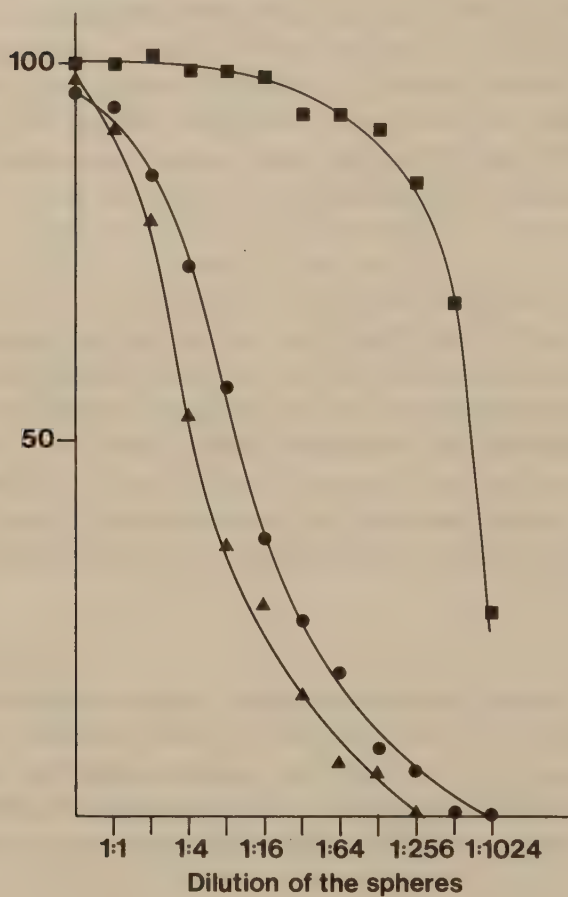


Fig. 4. Binding capacity of the bismuth spheres. Sedimentation of the spheres (●). Bismuth spheres incubated with gentle shaking for 2 h. (■). Sedimentation of bismuth spheres in samples containing ^{125}I -labelled rabbit IgG and 8 mg/l native rabbit IgG (▲).

GENERAL CONSIDERATIONS ON THE MATRIX

The attenuation capacity of the reagent is probably dependent on how closely the γ -radiating tracer can be adsorbed to the Bi_2O_3 crystals. Compared to the adsorber-attenuator reagent prepared by block-polymerization in agarose, the attenuation capacity of Bi_2O_3 was reduced by entrapment in starch spheres (fig.5). Although increasing amounts of bismuth oxide were entrapped in the starch spheres, the leakage could not be reduced below 15 %. The effective adsorption and attenuation properties were restricted to those of the surface of the spheres. Due to the high carbohydrate content of the spheres, 37 % dry weight, only a small fraction of the antigen can diffuse deeply into the spheres. The agarose matrix has a more porous structure and since the adsorber-attenuator reagent is disintegrated after polymerization, the Bi_2O_3 crystals are not coated with carbohydrates in the same way as the crystals in the starch spheres.

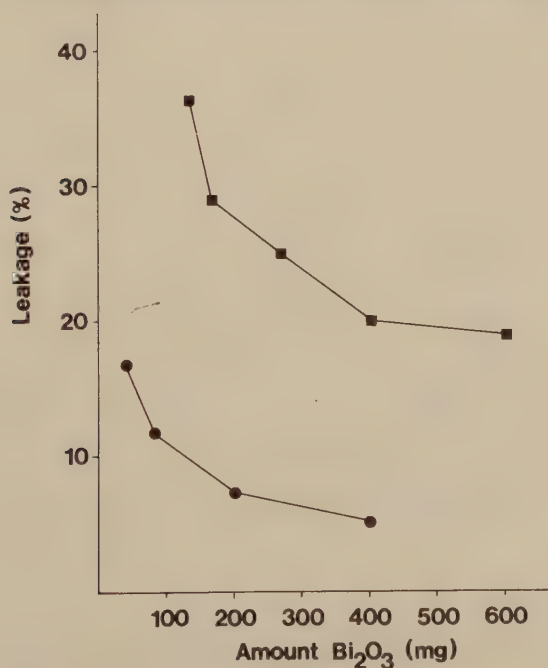


Fig. 5. Leakage of activity from pellets containing adsorbed ^{125}I - T_3 (25 pg) and varying amounts of Bi_2O_3 . Charcoal- Bi_2O_3 adsorber-attenuator reagent prepared by block polymerization in agarose (●) or by entrapment in starch spheres (■).

The larger pores and the higher mechanical stability of the agarose matrix makes it better to use in an adsorber-attenuator reagent. However, an even more porous support could improve the second antibody adsorber-attenuator reagent. An indication that the pore size of the agarose matrix is a limiting factor in the attenuator reagent is obtained from the amount of entrapped Bi_2O_3 that is needed to attenuate the γ -radiation from the ^{125}I -labelled rabbit IgG or from the B_0 value (70% binding) of ^{125}I -labelled human chorionic gonadotropin (HCG) (fig. 6). The ^{125}I -labelled rabbit IgG is quickly attenuated and no further attenuation is obtained after addition of more than 25 mg Bi_2O_3 per tube. More than five times this amount of Bi_2O_3 must be used to obtain the same attenuation of the ^{125}I -labelled HCG. The radioactive iodine atoms incorporated into the HCG molecule have a greater distance from the attenuator compared to the iodine atoms in the ^{125}I -labelled rabbit IgG. However, it is also possible that the immune complex penetrates the agarose matrix less, and thereby further increasing the distance from the attenuator.

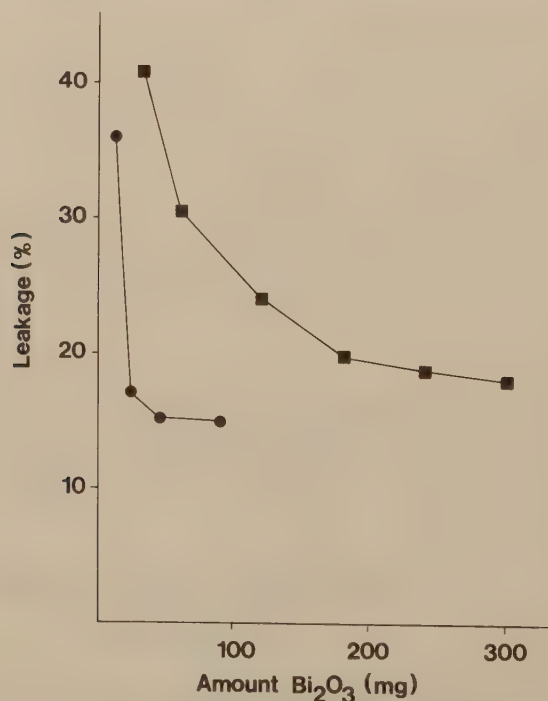


Fig. 6. Leakage of activity from the second antibody reagent after adsorption of ^{125}I -labelled rabbit IgG (●) or ^{125}I -labelled HCG incubated with rabbit serum against HCG (■).

The second antibody-attenuator reagent has like all other solid-phase reagents a tendency for non-specific adsorption. A bias in the result of the assay is obtained without pretreatment of the spheres with dextran and Tween 20 or dextran and addition of serum to the samples prior to addition of spheres (Paper II).

Another kind of mis-classification error was produced by the charcoal-attenuator reagents (Paper III). With both the agarose and the starch reagents a time dependent decrease in counts after sedimentation of for triiodothyronine (T_3) measured one hour (sedimentation time) after addition of the agarose-charcoal- Bi_2O_3 -reagent and the same calibration curve measured 3 hours later.

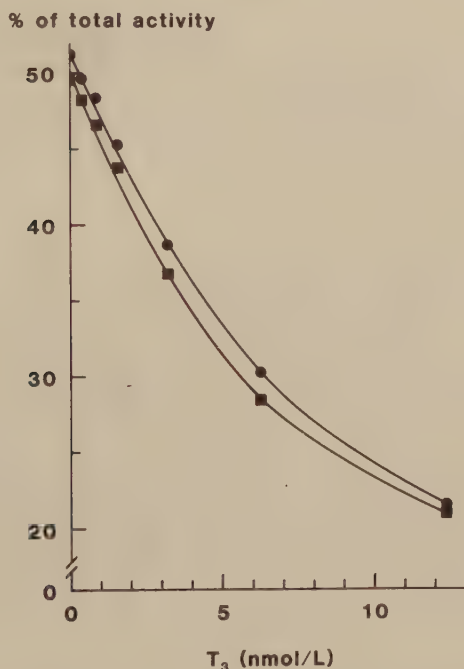


Fig. 7. Standard curve for a triiodothyronine (T_3) assay with agarose particles containing Bi_2O_3 and charcoal (●). The same calibration curve measured 3h. later (■).

The decrease in activity, or drift, was of the order of one per cent / hour of the activity in the tubes, but it introduced a much higher error in the amounts of T_3 in the samples estimated from the same calibration curve (Table II).

TABLE II
The effect of drift on measured amounts of T_3 in a sample

Time after measuring the calibration curve (min.)	Measured amounts of T_3 (nmol/l) ^a			
	Agarose reagent		Starch reagent	
	K1	K2	K1	K2
0	2.1	5.9	1.8	5.4
90			1.9	5.7
170	2.3	6.0	2.1	6.3
260			2.1	6.1
330	2.6	6.5		
540			2.4	6.7
780			3.0	7.5

^amean from triplicates.

K1: pooled serum control containing T_3 2 nmol/l

K2: pooled serum control containing T_3 6 nmol/l

The drift is due to a stripping effect on the antigen-antibody complex. After addition of the adsorber-attenuator reagent the supernatant contains no free antigen. This changes the equilibrium between antigen and antibody and some of the antigen-antibody complexes dissociate. The released antigen diffuses into the pellet and becomes adsorbed and attenuated. The change in equilibrium cannot be prevented, instead the released antigen can be stopped from reaching the attenuating pellet. This can be achieved by adding pure starch spheres or corn starch particles to the adsorber-attenuator reagents. After addition to the samples, the starch particles sediment slower and form a diffusion barrier on the top of the attenuating pellet. The addition of starch reduces the drift to nearly the same decrease in counts that is caused by the radio nuclide decay (Paper III). The same reduction of the drift was also obtained in a radioimmunoassay of digoxin.

CONCLUDING REMARKS

Immunoassays involve time consuming and laborious steps and several attempts to automate the performance of the assay have been made. Completely automated systems has been developed with homogeneous enzyme immunoassays and with an immunoassay system (PARCIA) that utilizes the aggregation of particles with coupled antibodies in the presence of the antigen (Cambiaso et al.,1977).

The use of an internal sample attenuator in radioimmunoassay circumvents the need for centrifugation and decantation of the samples. These two steps are the main obstacles when attempting to automate an immunoassay that utilizes a radioactive isotope as a tracer. The internal sample attenuator counting (ISAC) technique is easy to work with and the performance of the assay can easily be automated. Initial mis-classification errors like "drift" and non-specific binding have been circumvented. However, Bi_2O_3 can only be used as an attenuator in radioimmunoassays utilizing ^{125}I as a tracer. Other γ -emmiting tracers used in radioimmunoassays have a radiation energy that poorly fits the attenuator electrons of bismuth.

So far the ISAC technique has only been used as a one step procedure to separate the free from the bound fraction of the antigen after the primary incubation. An adsorber-attenuator reagent with a high attenuation capacity and a primary antibody coupled to the particles can be difficult to obtain. This kind of reagent will give a large excess of antibodies and thereby lower the sensitivity of the assay. An alternative to this would be to use a monoclonal antibody coupled to the attenuating spheres. After incubation of a monovalent antigen and the same monoclonal antibody, the spheres can be used to adsorb and attenuate the free fraction of the antigen.

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INTRODUCTION

The difference between the internal milieu of the cell and the external environment is maintained by the plasma membrane. In 1972 Singer and Nicolson proposed the fluid mosaic model of the cell membrane. This model is today accepted as the structure of the cell membrane. Briefly, the lipids are amphipatic molecules arranged in a lipid bilayer with the hydrocarbon chains of the lipids in the middle of the plasma membrane. The membrane proteins are also amphipatic and the non-polar regions are embedded in the hydrophobic interior of the membrane in a mosaic arrangement. The cell membrane is a fluid structure in which both the lipids and the proteins are able to perform translational movements within the bilayer. The components of the membrane are held in place only by non-covalent interactions and can be dissolved by detergents.

The lipids of the plasma membrane vary in their proportions between different cell membranes but contain mainly phospholipids, cholesterol and galactolipids. The major membrane phospholipids are phosphatidylcholine, phosphatidylethanolamine and sphingomyelin which all are neutral or positively charged at neutral pH. As an average, 5 to 20 per cent of the phospholipids in the plasma membrane are acidic. The main acidic phospholipids that are negatively charged in the plasma membrane are phosphatidylinositol, phosphatidylserine and phosphatidylglycerol.

Membrane proteins are differentiated into two groups, the peripheral or extrinsic proteins and the integral or intrinsic proteins. Some of the criteria that are used to differentiate the proteins are listed in Table I.

Table I

Criteria for peripheral and integral membrane proteins^a

	Peripheral Proteins	Integral Proteins
Dissociation from the membrane	High ionic strength Metal ion chelating agents	Detergents Organic solvents Chaotropic agents
Presence of lipids after solubilization	Free of lipids	Associated with lipids
Solubility	Soluble in buffers	Insoluble or aggregated in buffers without detergents
Examples	β_2 -microglobulin Spectrin	Histocompatibility antigens, Hormone receptors

^aFrom Singer, S.J., "The Molecular Organization of Biological Membranes", in Rothfield, L.I., (Ed), The Structure and Function of Biological Membranes, New York, Academic Press, Inc., 1971, p.145

Peripheral membrane proteins are only loosely attached to the membrane and can be solubilized without disintegration of the plasma membrane. After solubilization, these proteins are free of lipids and are soluble without the presence of detergents. Integral membrane proteins are deeply inserted into the lipid bilayer and sometimes span the whole lipid bilayer. To solubilize these kinds of proteins the whole plasma membrane has to be dissolved by organic solvents or detergents. After solubilization the proteins are usually associated with lipids and detergents have to be present to maintain the proteins in non-aggregated forms. A schematic presentation of the structure of two integral membrane proteins, the histocompatibility class I antigen and the insulin receptor, is shown in figure 1.

The histocompatibility class I antigen contains two polypeptides, a heavy chain of 44,000 daltons and a light chain of 12,000 daltons. The heavy chain is a glycoprotein and spans the cell membrane. The light chain is called β_2 -microglobulin and is non-covalently associated with the heavy chain (Trädgård et al., 1979).

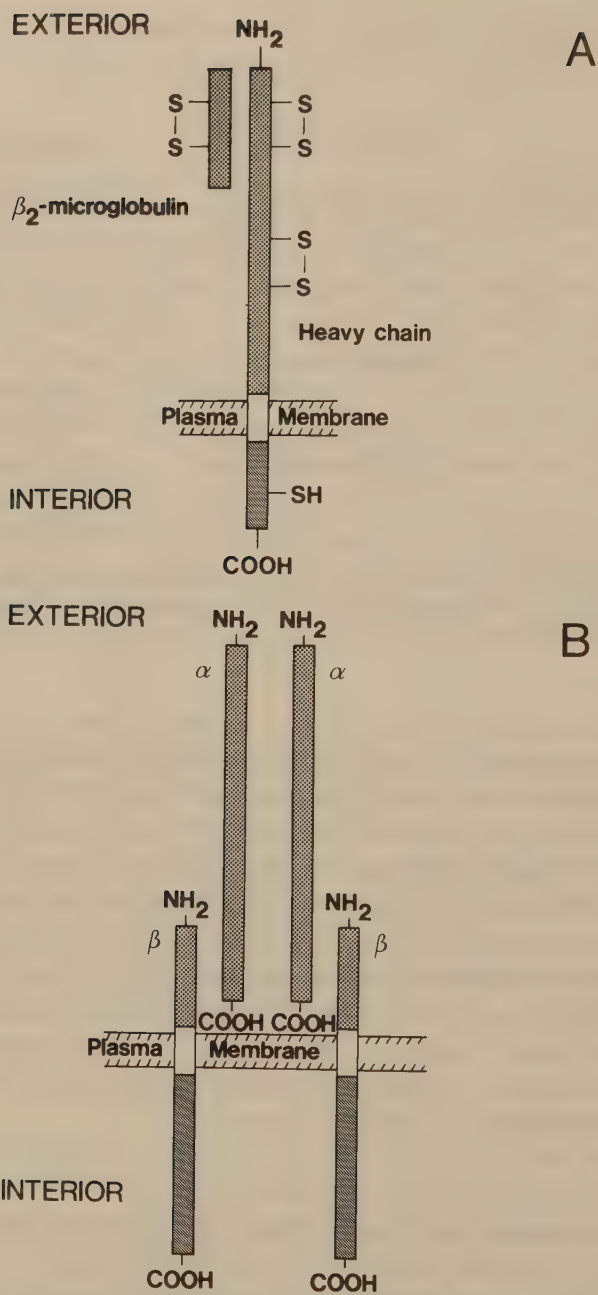


Fig. 1. A. Schematic presentation of the major histocompatibility class I antigen (MHC-1). From Stominger et al., 1976
 B. Schematic presentation of the insulin receptor. From Ullrich et al., 1985

The insulin receptor of 350,000 - 400,000 daltons is composed of two α -subunits (120,000 - 130,000 daltons) and two β -subunits (90,000 daltons), linked by disulphide bounds (Cuatrecasas,1973, Siegel et al.,1981). The α -subunits contain the binding site of insulin although the β -subunit amino terminus may also be involved (Jacobs et al.,1979). The insulin receptor has an insulin-dependent tyrosine kinase activity that catalyses phosphorylation of both the β -subunit and exogenous peptides and proteins (Grigorescu et al.,1983, Lauret and Rosen,1983). The β -subunit has an ATP-binding site and probably contains the protein kinase activity (Van Obbergehen et al.,1983). The complete amino-acid sequence of the human insulin receptor and the precursor of the receptor are known (Ullrich et al.,1985). Only the β -subunit has hydrophobic stretches long enough to span the plasma membrane and a structure of the receptor was proposed by Ullrich et al., 1985 with the α -subunit exclusively on the surface of the cell, held in place by disulphide links to the β -subunits (fig.1).

The second part of this thesis, based on papers IV - VI, deals with the use of lipid vesicles as tools to investigate the structure and function of integral membrane proteins such as the histocompatibility antigen and insulin receptor. Papers IV and V are concerned with the detection of membrane proteins incorporated into liposomes, while paper VI shows a method to introduce new membrane proteins into the plasma membrane of viable cells.

IMMUNOASSAY OF MEMBRANE PROTEINS

Peripheral membrane proteins are soluble in neutral buffers and do not require detergent to remain in a non-aggregated form. Conventional immunoassay techniques can be applied to peripheral membrane proteins and if the proteins are shed into the serum, the assay can have a clinical relevance. Examples of this are the assays for β_2 -microglobulin and carcino embryonic antigen (CEA).

Integral membrane proteins are deeply inserted into the cell membrane having a hydrophilic part protruding from the membrane surface, sometimes at both the external and internal surfaces, and a hydrophobic part hidden in the bilayer. The hydrophilic parts of the integral membrane proteins can be extracted by proteolytic digestion (Shimada and Nathenson, 1967). The liberated parts of the membrane proteins are soluble and conventional immunoassay techniques can be performed. However, the hydrolytic step may generate structural changes in the epitope.

The complete molecule of the membrane protein is obtained by detergent solubilization of the cell membrane. After this procedure, the membrane proteins can be isolated in the presence of detergent. The efficiency of an immunological method used to identify isolated membrane proteins may however be influenced by the way the protein is presented.

The detergent can be removed if the membrane proteins are inserted into artificial lipid vesicles, liposomes. This offers the potential for using such liposomes as targets in immunoassays.

LIPOSOMES

Phospholipids spontaneously form vesicles called liposomes, consisting of a bilayer of lipids, after hydration above the transition temperature of the phospholipids. Two main types of liposomes can be distinguished, the large uni- or multilamellar liposomes and the small unilamellar liposomes.

Preparation of liposomes

A. Large liposomes

1. Multilamellar vesicles

Bangham et al.(1965) were the first to describe multilamellar liposomes. The liposomes were prepared by deposition of a lipid film from an organic solvent onto the walls of a container, followed by agitation in an aqueous solution.

2. Large uni- or oligolamellar vesicles

Ether infusion (Deamer and Bangham, 1976).

The liposomes are formed by injection of a solution of lipids in ether into an aqueous solution at a temperature high enough for the rapid evaporation of the solvent.

Calcium-induced fusion (Papahadjopoulos et al., 1975)

Acidic phospholipids form cochleate cylindrical structures with calcium ions, which are transformed into large unilamellar vesicles after addition of chelating agents.

Reverse-phase-evaporation (Szoka and Papahadjopoulos, 1978)

A homogeneous emulsion of an aqueous phase and an organic phase containing phospholipids is prepared by sonication. Removal of the organic solvent under reduced pressure results in the formation of large unilamellar and oligolamellar liposomes.

B. Small unilamellar liposomes.

Sonication (Papahadjopoulos and Miller, 1967)

Multilamellar liposomes are prepared and after sonication with a probe or bath sonicator small unilamellar liposomes are formed. The mean diameter of these liposomes are about 50 nm.

Ethanol injection (Batzri and Korn, 1973)

The technique is, in principle, similar to the ether injection method. Instead of ether, ethanol is used to solubilize the phospholipids.

Detergent removal

Mixed micelles of phospholipids and detergents are formed in an aqueous solution. Small unilamellar liposomes are formed after removal of the detergent either by dialysis (Kagawaga and Racker, 1971), gel-filtration (Curman et al., 1978) or adsorption to polystyren beads (Volsky et al., 1979).

In order to incorporate membrane proteins into the lipid bilayer of the liposome without exposing the membrane proteins to organic solvents, only the calcium-induced fusion or the detergent removal methods can be used. Integral membrane proteins are usually purified after detergent solubilization of the cell membranes and the most obvious method to incorporate these membrane proteins is to use the detergent removal method. However, the detergent can not be completely removed and the liposomes will be mixed liposomes containing lipids, membrane proteins and detergent. Usually a few mole per cent detergent (mole detergent per mole phospholipid) remain liposome associated.

DETERGENTS

Detergents used in liposome preparation can be divided into ionic and nonionic classifications. Examples of ionic detergents are the bile salts, sodium cholate and sodium deoxycholate, while nonionic detergents include Triton X-100 and β -D-octylglucoside. The critical micelle concentration (CMC), which is the concentration of the detergent above which it forms micelles, and the aggregation number, which determines the size of the detergent micelle, must be considered when liposomes are prepared by the detergent removal method. The properties of some commonly used detergents are listed in Table II. The ionic detergents listed have a high CMC and a low molecular weight of micelles. These detergents can therefore be removed by dialysis or gelfiltration. However, ionic detergents affect the structure of many membrane proteins. Sodium deoxycholate, for example, inactivates the binding capacity of the insulin receptor.

Table II

Properties of some commonly used detergents in liposome preparation ^a

Detergent	Mol wt	Aggregation number	Micellar mol wt	CMC (mM)	Approx. halftime for dialysis (h)
Sodium cholate	431	2-4	900-1800	13-15	3
Sodium deoxycholate	415	4-10	1700-4200	4-6	10
Triton X-100	642	140	90,000	0.24	50
β -D-octylglucoside	292			25	2

^aData from Allen, J.M., "Removal of detergent and solvent traces from liposomes", in Gregoriadis, G. (Ed), Liposome Technology, vol I, Ch. 8, CRC Press Inc., Boca Raton, Florida

Removal of the detergent

Nonionic detergents have, with few exceptions such as the octyl-glucoside, low CMCs and high aggregation numbers. This makes it difficult to remove the detergents by dialysis or by gel filtration during liposome formation.

Several membrane proteins are extracted from cell membranes by detergents such as Triton X-100. In order to reconstitute these proteins into liposomes a polymeric adsorbent or replacement of the detergent has to be used. The polymeric adsorbent, such as Biobeads SM-2 or Amberlite XAD-2, can be added to the buffer used in the dialysis procedure to speed up the removal of detergent. Another way is to pass the detergent-phospholipid solution over a column of the resins. As a third possibility the resins can be added directly to the detergent-phospholipid solution. Dialysis against the adsorber (Volsky et al., 1979) is time-consuming although the time is greatly reduced compared to when the beads are omitted. The adsorption of membrane proteins is very high if the resins are packed in a column. Binding of membrane proteins to the beads after addition to the detergent-phospholipid solution (Vainstein et al., 1984 and Paper V) occurs, especially when low amounts of proteins such as purified hormone receptors are used. However this can be avoided by adding albumin to the detergent solution before addition to the adsorber.

Incorporation of proteins from the major histocompatibility complex into liposomes has been described (Curman et al.,1978, Engelhard et al.,1978, Littman et al.,1979). It was shown that the major mouse H-2 and human HLA antigens expose their antigenic determinants externally when they are incorporated into small liposomes. This offers the potential for using such liposomes as targets in immunoassays.

Complement mediated lysis

Liposomes have been used as targets both for antibody binding and for complement lysis. Complement-mediated damage of the liposomes causes permeability of the lipid layer to relatively large molecules. This technique was first described by Kinsky,1972, using glucose as the entrapped marker. Other markers such as enzymes (Kataoka et al.,1973) and spin-labelled molecules (Chan et al.,1978) have also been used.

Agglutination of liposomes

Another way of utilizing liposomes as targets in immunoassays has been to agglutinate the liposomes with antibodies (reviewed by Alving and Richards,1983). Paper IV describes the detection of membrane antigens incorporated into liposomes by this method. Partially purified rat histocompatibility antigen (RT-1) was incorporated into liposomes and aggregated by incubation, first with a primary antiserum, followed by a secondary antiserum. The amount of precipitated antigen was quantified either by a ^{125}I - or a fluorescence-labelled lipid incorporated in the liposomes. The present performance of the assay has been altered from the method described in paper IV where mainly a ^{125}I -labelled lipid was used as a tracer. Due to the γ -emission from the tracer, the liposomes were easily detected in the precipitate.

The tracer routinely used today is a conjugate between 7-chloro-4-nitrobenz-2-oxa-1,3-diazol (NBD-chloride) and phosphatidylethanolamine. The fluorescent lipid is commercially available and is also easily synthesized (Monti et al.,1978). The conjugate has the advantage of high solubility and high fluorescence in ethanol. The solubility in ethanol makes the NBD-phosphatidylethanolamine (NBD-PE) able to be extracted from the immuno-precipitate by mixing the precipitate with ethanol. After extraction of the NBD-PE the immuno-precipitate sediments rapidly and the fluorescence can be measured without interference. Using NBD-PE, the detection of the amounts of liposomes in the immuno-precipitate is nearly as simple as using a γ -emitter as a tracer.

Lipid exchange

The use of NBD-PE makes it possible to observe the lipid exchange between liposomes and serum (Eriksson and Mattiasson, 1984). The NBD-PE conjugate forms self-quenched aggregates, probably micelles. After removal of the detergent, only a few per cent of the total fluorescence can be detected from the self-quenched NBD-PE conjugate. However, when whole serum is added to self-quenched NBD-PE, increased fluorescence can be detected. No increase is observed when an immunoglobulin fraction is added (fig.2).

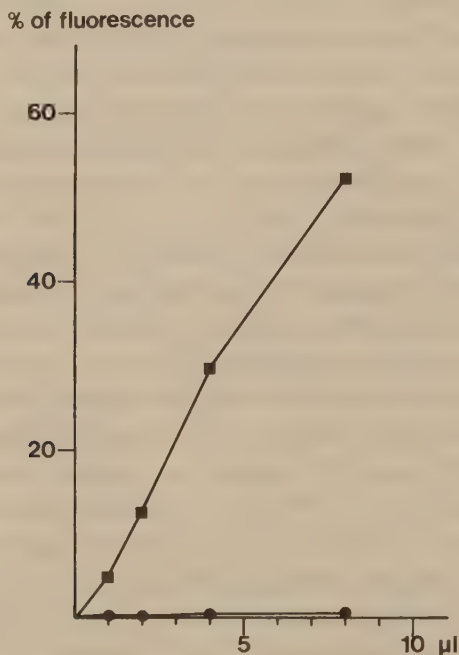


Fig. 2. Release of fluorescence from self-quenched NBD-PE after addition of varying amounts of a rabbit anti rat IgG serum (■) or an immunoglobulin fraction of the rabbit anti rat IgG (●).

Lipid exchange probably caused the decrease of precipitated activity upon prolonged incubation with rabbit anti-rat IgG serum which was observed in paper IV. To avoid a decreased sensitivity of the assay and the possibility of determining a positive sera as negative, only the immunoglobulin fraction is now used in the secondary precipitation of the liposomes (Eriksson and Mattiasson, 1984).

So far no effect of lipid exchange has been observed from primary serum or from ascites. However, care has to be taken in selecting the

the pool of normal sera used to co-precipitate the liposomes and extremely lipid rich sera should be avoided in the pool.

Lipid composition

The most sensitive assay should be obtained if the membrane proteins were incorporated into liposomes containing only labelled lipids and thereby giving the antigen a very high specific labelling. However, this can not be achieved with NBD-PE as the labelled lipid. If the detergent is removed from solutions containing fixed amounts of NBD-PE, fixed amounts of affinity purified rat major histocompatibility complex class 1 (rat MHC-1) and decreasing amounts of a total lipid extract from rat liver, an increased non-specific precipitation is obtained (fig. 3). The non-specific precipitation varies with different ascites and sera and can not be inhibited by pre-incubation with bovine albumin or human IgG (25 times in excess of the IgG content of the ascites used as primary antibody).

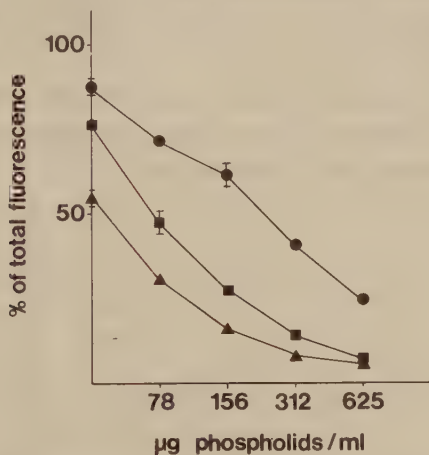


Fig. 3. Immuno-precipitation of MHC-1 containing lipid vesicles prepared by the Biobead method using varying amounts of a total lipid extract from rat liver. Precipitation with a monoclonal antibody against rat MHC-1 (●) or precipitation with a monoclonal antibody against human MHC-1 (▲). Precipitation of non MHC-1 containing lipid vesicles by the monoclonal antibody against rat MHC-1 (■).

A more pronounced non-specific precipitation is observed with NBD-PE in combination with charged lipids. A mixture of NBP-PE and a neutral phospholipid, phosphatidylcholine, gives a lower background but still a high specific precipitation (fig. 4).

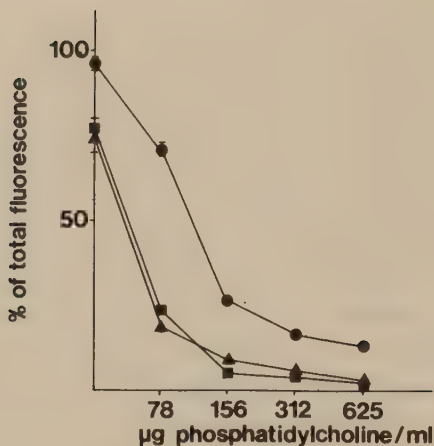


Fig. 4. Immuno-precipitation of MHC-1 containing lipid vesicles prepared by the Biobead method using varying amounts of phosphatidylcholine. Precipitation by a monoclonal antibody against rat MHC-1 (●) or by a monoclonal antibody against human MHC-1 (▲). Precipitation of non MHC-1 containing lipid vesicles by the monoclonal antibody against rat MHC-1 (■).

A specific precipitation can still be detected with a 1:1600 dilution of an ascites against rat MHC-1 using a phosphatidylcholine concentration of 156 µg/ml during liposome formation (fig. 5).

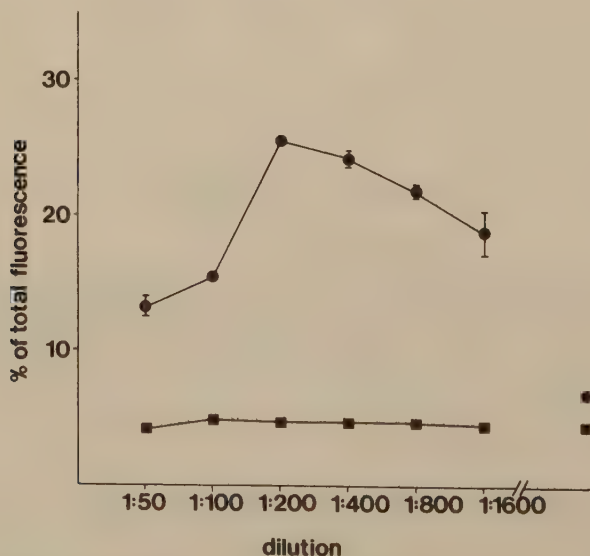


Fig. 5. Immuno-precipitation by a dilution series of an ascites against rat MHC-1. Precipitation of liposomes containing affinity purified rat MHC-1 (●) and precipitation of liposomes without incorporated rat MHC-1 (■).

BINDING OF LIPOSOMES TO CELLS

A commonly used method to detect binding of ligands to detergent solubilized membrane receptors is the polyethylene glycol (PEG) assay described by Cuatrecasas, 1972. The final PEG concentration used in this method to precipitate the membrane receptor-ligand complex is 10 % (w/w). This concentration of PEG is the limitation of the method since ligands precipitating in 10 % PEG can not be used.

A possibility to detect binding of ligands which precipitate in 10 % PEG is described in paper V. After incorporation of membrane proteins into liposomes, the liposomes were bound to carrier cells to facilitate the separation of membrane receptor-ligand complexes from free ligand. Attachment of the liposomes to the carrier cells was obtained by the lectin concanavalin A, supposed to act as a "bridge" between carbohydrates on the carrier cells and glycolipids in the liposomes.

However, binding of liposomes to the cells was mainly due to interactions between the lectin and the carbohydrate part of the incorporated membrane protein and not to the glycolipids incorporated into the liposomes (Paper V).

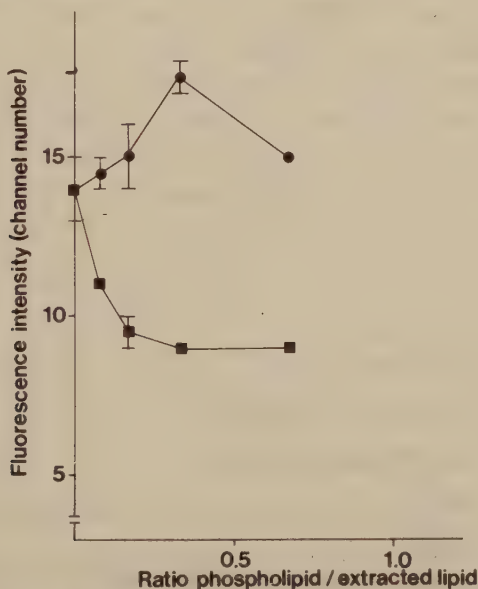


Fig. 6. Lectin mediated binding of rat MHC-1 antigen to cells after preparation of liposomes containing glycolipids extracted from *Micrococcus lysodeikticus* and various amounts of phosphatidylcholine (■) or phosphatidylethanolamine (●).

The amount of liposomes attached to the cells was dependent on the lipid composition of the liposomes (fig. 6). Increasing amounts of rat MHC were bound to the cells when the antigen was incorporated into liposomes containing the positively charged lipid phosphatidylethanolamine. Later results indicate that the increased attachment of phosphatidylethanolamine containing liposomes was due to a higher affinity of the liposomes for the cells. The higher affinity is interpreted as the combination of the lectin mediated binding and the electrostatic interaction between the liposomes and the cells.

Liposomes containing inserted membrane proteins are also precipitated by 10 % PEG. Figure 7 shows the binding of ^{125}I -insulin to affinity purified receptor and varying amounts of phosphatidylcholine after removal of the detergent Triton X-100 by addition of Biobeads SM-2 as described in paper V.

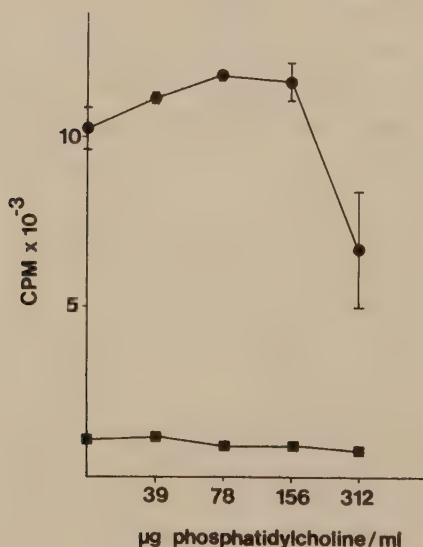


Fig. 7. Binding of ^{125}I -insulin to affinity purified receptor incorporated into liposomes.

Insulin receptor, affinity purified according to Fujita-Yamaguchi et al., 1983, was incorporated into phosphatidylcholine vesicles and incubated overnight at 4°C with ^{125}I -insulin (●) or with ^{125}I -insulin in the presence of 25 $\mu\text{g/ml}$ unlabelled insulin (■).

A high binding capacity of the receptor is obtained without any addition of phosphatidylcholine, indicating that the detergent cannot be completely removed from the receptor by the Biobeads. Higher concentrations of phosphatidylcholine during liposome formation reduce the binding capacity of the receptor.

The optimal phospholipid concentration during liposome formation using Biobeads is lower than the concentration used during formation of liposomes by gel filtration on Sephadex G 25. In paper V, affinity purified insulin receptor was incorporated into liposomes by the Biobead method, using an optimum lipid concentration for the gel filtration procedure. The Biobead method has later been shown to have an optimum lipid concentration of ten times less than the amount used. The higher lipid concentration reduces the binding capacity of the receptor (fig.7) and in paper V only a weak binding of insulin or none at all was observed after attachment of these liposomes to carrier cells.

Attachment of liposomes to cells can be used as a method to detect binding of ligands such as antibodies or hormones to membrane components incorporated into liposomes. The attachment of liposomes to the surface of cells is also the first step towards introducing new membrane components into the cell membrane. Paper VI describes a method to introduce new membrane proteins after affinity attachment of liposomes to the cells, prior to addition of PEG to obtain conditions for fusion of the cells and liposomes.

Liposomes have important potential applications in cell biology. Integral membrane proteins can be solubilized and purified in the presence of detergents. However, the detergent has to be present in all the steps to avoid aggregation and precipitation of the proteins. The detergent can be replaced by phospholipids and the membrane proteins are spontaneously incorporated in the lipid bilayer of the liposomes. Once incorporated into liposomes, the membrane proteins can be added to living cells without any toxic effects by the detergent.

Liposome-cell interactions provide models of physiological processes in the cell membrane and liposomes can be used as a tool to introduce exogenous lipids and membrane proteins into the plasma membrane of living cells.

The various ways by which liposomes can interact with cells are schematically shown in figure 8.

Stable adsorption of intact liposomes to the cell surface can be mediated by non-specific electrostatic or hydrophobic forces. The adsorption can also be maintained by specific components such as antibodies or lectins (paper V and VI) present at the vesicle or cell surfaces.

Endocytosis is the uptake of intact liposomes which are presumably delivered to the lysosomes.

Fusion between cells and liposomes results in a mixing of the lipid components and a release of the liposome content into the cytoplasm of the cell.

Lipid exchange transfers individual lipid molecules between the liposomes and the cells without transfer of the aqueous content of the liposomes.

The schematic presentation above divides the cell-liposome interactions into four different groups. Addition of liposomes to cells results in cell-liposome association and a major problem is to determine to what degree the observed association is due to a particular mechanism.

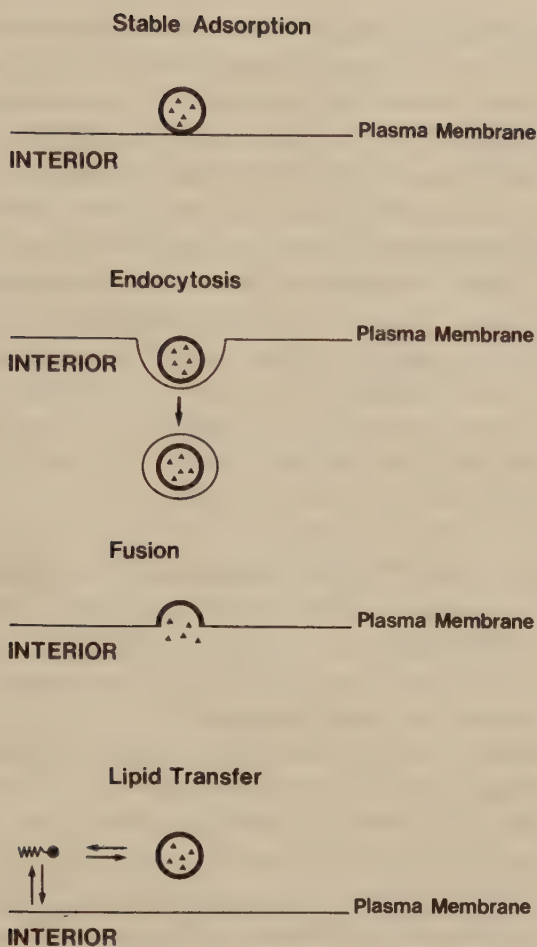


Fig. 8. Possible mechanisms of interaction between liposomes and cells. From Pagano and Weinstein, 1978

New lipids can be introduced in the cell membrane by lipid exchange between cells and liposomes. The mechanism of lipid exchange has been demonstrated for different phospholipids and cholesterol (Pagano and Huang, 1975). Cholesterol is transferred between cells and liposomes by transfer of mass (Bruckdorfer et al., 1969). The transfer of phospholipids may have a lipid specificity and no net transfer of mass (Sandra and Pagano, 1979). However, the lipid specificity cannot distinguish between phosphatidylethanolamine and the same lipid with a hapten covalently bound to the amino group (Schroit and Pagano, 1981).

In order to introduce membrane proteins into the recipient cells, fusion of cells and liposomes is required. Fusion between cells and liposomes can be produced by glycoproteins from Sendai virus envelopes (Prujansky-Jakobovits et al.,1980, Hale et al.,1980, Volsky et al.,1981), polyethylene glycol (Baird and Henkart,1983, Balakrishnan et al.,1983, Paper VI) or divalent ions such as Ca^{2+} (Eytan and Eytan,1980). Evidence that fusion has occurred is that the recipient cells can be lysed by complement and specific antibodies against the introduced membrane component (Prujansky-Jakobovits et al.,1980), or that they can be lysed by antigen specific T-lymphocytes (Hale et al.,1980). A third possibility is that the recipient cells can be stimulated by chemical messengers mediated by the introduced membrane component.

After attempts to obtain fusion between cells and liposomes, two main questions are how much of the membrane-associated, liposome-derived component is really inserted in the cell membrane and not merely adbed to the cell surface and for how long the introduced components will remain exposed on the cell membrane.

Different methods, such as labelled antibodies or labelled membrane proteins have been used to examine for how long time after fusion the introduced membrane proteins remain associated with the recipient cells. The half-life of the introduced proteins has been reported to range from less than one hour up to several days. The discrepancy may be due to introduction of membrane proteins which differ in their rate of turnover in the native cell membrane. However, in some studies it may also be due to the use of a detection method that cannot differentiate between membrane proteins inserted into the cell membrane and proteins adsorbed to the cell surface. Methods used to investigate the half-life of introduced membrane proteins must be correlated to one of the earlier mentioned criteria used as evidence for fusion between cells and liposomes.

In paper VI rat MHC was introduced into human lymphocytes after PEG induced fusion with liposomes. After fusion the human lymphocytes had an antigen density of about 10 % of the density found on a rat lymphocyte. Immediately after fusion and washing of the human lymphocytes,

the cells could be lysed with complement and antibodies against rat MHC. One hour later no lysis of the cells could be obtained. These results correlate well with the antigen density on the lymphocytes determined with a flow cytometer (Paper VI). Half-life of introduced membrane proteins of the same magnitude as the rat MHC antigens has also been reported after transfer of the thyrotropic hormone receptor (Balakrishnan et al., 1983). In this report the recipient cells could be stimulated by thyrotropin until approximately 100 minutes after fusion.

The introduction of new membrane components into living cells by cell/liposome fusion provides new opportunities for investigation of the function of the cell membrane. An obvious limitation of the method is the elimination of the introduced membrane proteins and ways to prolong the half-life must be further investigated.

CONCLUDING REMARKS

Part two of the thesis describes the use of liposome technology to investigate the structure and function of integral membrane proteins. The hydrophobic character of parts of the integral membrane proteins often presents particular problems in the laboratory work and a special methodology using detergents has to be adopted. The developments during the last years of the monoclonal antibody and cell culture techniques provide a possibility to affinity purify microgram or even up to milligram quantities of a specific membrane protein. After isolation of the membrane protein the detergent used to keep the membrane protein in solution can be replaced by phospholipids and the membrane protein becomes incorporated into the liposome bilayer. The incorporation of membrane proteins into liposomes restores the native micro milieu to the membrane protein, with its surroundings of lipids, and provides the possibility of restoring the biological functions of the membrane proteins.

The liposome technology makes it possible to create an artificial cell membrane with known lipid composition. Different affinity purified membrane proteins can be incorporated into the same liposome to study their biological functions. The concept can be used to study the molecular events in the cell membrane when a signal is transferred through the cell membrane by a chemical messenger. All transmembrane signals are probably mediated by a transformation change in the receptors' three dimensional structure leading to an association between the receptor and other membrane components. However, only a limited amount of membrane components can be introduced into the liposomes without obtaining a too complex system. Incorporation of a membrane protein or proteins into a cell can be obtained by cell/liposome fusion. This methodology provides a tool to study the effects of a very complex micro milieu on an isolated membrane protein.

The use of liposomes as drug carriers and as immunological adjuvants are well known areas of liposome technology. This thesis has focused on another application of liposome technology. The use of liposomes as a tool to study the structure and function of integral membrane proteins.

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USE OF AN INTERNAL SAMPLE ATTENUATOR IN RADIO-IMMUNOASSAY. ASSAY OF TRIIODOTHYRONINE (T_3) USING STARCH PARTICLES CONTAINING ENTRAPPED CHARCOAL AND BISMUTH OXIDE IN COMBINATION WITH FREE ANTIBODIES

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A radioimmunoassay for triiodothyronine involving no separate washing or separation steps is described. By using an internal sample attenuator, bismuth oxide, co-immobilized with the sorbent, charcoal, for the non-bound fraction of T_3 , a system was designed in which a suspension of starch spheres containing the sorbent and the attenuator was added after the immunological reaction had taken place. The particles sedimented and the whole test tube was counted in a gamma-counter.

The coefficient of correlation between the results obtained with the present method and those from conventional procedures was 0.993.

INTRODUCTION

Competitive binding assays usually involve separation of bound and free ligand. The various separation and washing steps often impair the precision of the assay. Furthermore, these steps are often performed by hand and are laborious. Less time-consuming procedures are therefore desirable.

Most successful simplifications of the experimental procedure in immunoassays have so far been made in enzyme immunoassays; homogeneous assays have been developed (Rubenstein et al., 1972; Burd et al., 1978; Mattiasson and Borrebaeck, 1980). Modification of the immunoassay in this way was relatively easy from a theoretical point of view since many methods are available for modifying the enzyme activity. The problem that had to be solved was how to modify only the bound enzyme or only the free enzyme. Several solutions to this problem are nowadays available.

For a long time radioimmunoassay was regarded as having an inherent disadvantage; the decomposition of the radioactive isotope could not be manipulated, and this prevented a development parallel to that of enzyme immunoassays. However, the use of an internal sample attenuator in combination with the solid phase making it possible to shield all, or at least most, of

the radioactivity bound to the solid phase has recently been reported (Thorell, 1977). This was done by taking advantage of the fact that most high density materials are only poorly penetrated by gamma radiation of ^{125}I (27–35 keV).

The immunoreactants studied were T_3 and anti- T_3 antibodies. After the immunoreaction had taken place with both reactants in free solution, a solid phase containing adsorbing material as well as the attenuator was added. The T_3 not bound by the antibodies was adsorbed to, and sedimented with, the particles.

Provided proper attenuation occurs in the solid phase it would be possible to assay the amount of T_3 bound by the free antibodies with no further separation.

MATERIALS

Starch, 'soluble' AnalR batch no. 2978430, was obtained from BDH Chemicals Ltd., Poole, U.K. The emulsifier, GAFAC PE-510, came from Svenska GAF, Stockholm, Sweden. Charcoal 'zur Analyse' and bismuth oxide of 'reinst' grade was obtained from Merck, Darmstadt, F.R.G. Prior to use, the bismuth oxide crystals were ground to an average particle size of $1\text{ }\mu\text{m}$. Agarose type 1 and 8-anilino-1-naphthalene-sulfonic acid (ANS) were purchased from Sigma, St. Louis, MO, U.S.A. and Tween 20 from Technicon Chemicals Co., Belgium. Dextran T 70 came from Pharmacia Fine Chemicals, Uppsala, Sweden. Bovine serum albumin (BSA) fraction V was obtained from Armour Pharmaceutical Company Ltd., U.K.

$[\text{}^{125}\text{I}]\text{Triiodothyronine}$ ($[\text{}^{125}\text{I}]\text{T}_3$) NEX-110, 100–150 Ci/g was purchased from New England Nuclear, Boston, MA, U.S.A. Triiodo-L-thyronine 'puris' was obtained from Henningberlin, Berlin, F.R.G. T_3 -free serum was prepared by adsorption to activated charcoal (Thorell and Larsson, 1978). Antiserum against T_3 was raised in rabbits by immunization with triiodo-L-thyronine coupled to human serum albumin with carbodiimide (Thorell and Larsson, 1978), using Freund's complete adjuvant. Human serum samples of T_3 were obtained from the routine analytical laboratory at Malmö General Hospital. The tubes in the assays were Ellerman tubes $11\text{ mm} \times 55\text{ mm}$ and Trombotest tubes $14\text{ mm} \times 81\text{ mm}$.

Barbital buffer, 0.075 M, pH 8.6, containing 0.05% sodium azide as preservative was obtained by dissolving 2.07 g barbituric acid, 13.14 g sodium barbiturate and 0.5 g sodium azide per liter.

Equipment used. The stirrer in the bead formation process was a Heidolph type RZR 1 and the sonicator was a A 350 G, Ultrasonic Ltd., Yorks., U.K. The bismuth oxide crystals were ground with an Aeroplex-Spiralstrahlmühle 200 AS (Alpine AG, Augsburg, F.R.G.) for 16 min to a maximal size of $5\text{ }\mu\text{m}$. A gamma-counter, LKB Wallac 1280 Ultrogamma, LKB, Bromma, Sweden was used for measuring the samples.

METHODS

Preparation of microspheres

Microspheres were prepared using a slight modification of a previously published method (Mosbach and Schröder, 1979). Twelve grams of bismuth oxide and 0.5 g of charcoal were mixed with 10 ml of water containing 0.25% (w/v) Tween 20. The slurry was sonicated with a probe sonicator 350 W for 1 min. Six grams of starch were added to the suspension and the slurry was heated to 95°C and then poured rapidly into a 3-necked flask containing toluene with the dissolved emulsifier, GAFAC PE-510 2% (w/v), stirred at 0°C with a speed of 900 rev/min. The beads produced were on average 10–40 μm in diameter. They were suspended in 200 ml of acetone and left to sediment for 5 min. The supernatant was then aspirated and the same procedure was repeated 4 times and then 4 times each with 400 ml of water. In the last step the spheres were left to sediment for 30 min. Finally the spheres were suspended in barbital buffer, 0.075 M, pH 8.6, containing 0.25% BSA (w/v) and 0.05% dextran T 70 (w/v). The suspension of the spheres used for assay was prepared from 15 g of wet spheres suspended in 15 ml of the barbital-BSA buffer.

T₃-assay

The assay was routinely performed in the following way: 200 μl [^{125}I] T₃ (125 pg/ml with 1 mg/ml ANS) and 200 μl of anti-T₃-serum, diluted 1:1000 in barbital-BSA buffer was added to 100 μl of standard T₃ (0, 0.38, 0.77, 1.54, 3.08, 6.15, 12.3 nmol/l) diluted with serum free of T₃, or 100 μl of the sample to be analyzed. The samples were mixed for 2 sec and incubated overnight at 4°C. The next day, 1 ml of starch sphere suspension containing charcoal and bismuth oxide was added to the assay tubes. To facilitate pipetting of the suspension, the outer tip of the plastic pipette was cut off, thereby widening the opening. The samples were mixed for 2 sec and then left to sediment for 1 h. The tubes were measured in a gamma-counter, i.e. without further separation or washing. The assay was performed in tubes with an inner diameter of 12 mm. Preliminary experiments had shown a wider range of variation of results obtained when using tubes of smaller diameter.

RESULTS AND DISCUSSION

In this study, carbohydrate polymers were used because they showed only a low tendency to non-specific adsorption. The polymers chosen were agarose and starch. Since the purpose of this study was to develop a system that, after the immunological reaction had taken place, was mixed only once more when the attenuator was added, it was necessary to develop small particles capable of sedimenting within a practically feasible period but at the same time slowly enough to permit adsorption of the antigen not

immunologically bound, i.e. the free T_3 molecules.

For this purpose agarose-bismuth oxide-charcoal aggregates were prepared. Bead polymerization according to previously published procedures (Hjertén, 1962) was tried first, but it was soon realized that the agarose beads would be too large and would sediment too rapidly. Furthermore, when decreasing the bead size by increasing the amount of emulsifier, the range in size variation was very wide. In order to obtain small spherical particles, starch was used (Mosbach and Schröder, 1979). When a solution of starch, heated to 95°C, was poured into an ice-cold organic phase consisting of toluene and GAFAC PE 510, small particles with a narrow range of variation of particle size were obtained. In all the experiments 37.5% dry weight of starch was used when preparing the starch particles. Fig. 1 shows a photomicrograph of the beads used. At first it was not possible to produce small particles because the bismuth oxide crystals were too large, but grinding of the crystals into smaller pieces gave starch spheres of suitably small size containing bismuth oxide.

A crucial point when using entrapped charcoal is the adsorptive capacity of such preparations. Fig. 2 shows that the binding capacity is high and that more than 90% of free T_3 was adsorbed. However, compared with free

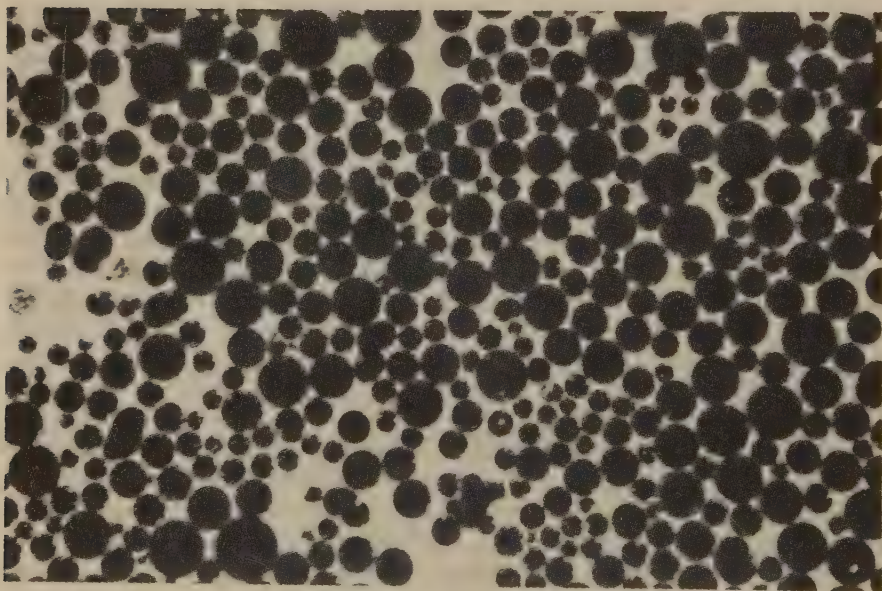


Fig. 1. Microphotograph of starch-charcoal-bismuth oxide spheres.

% of total activity

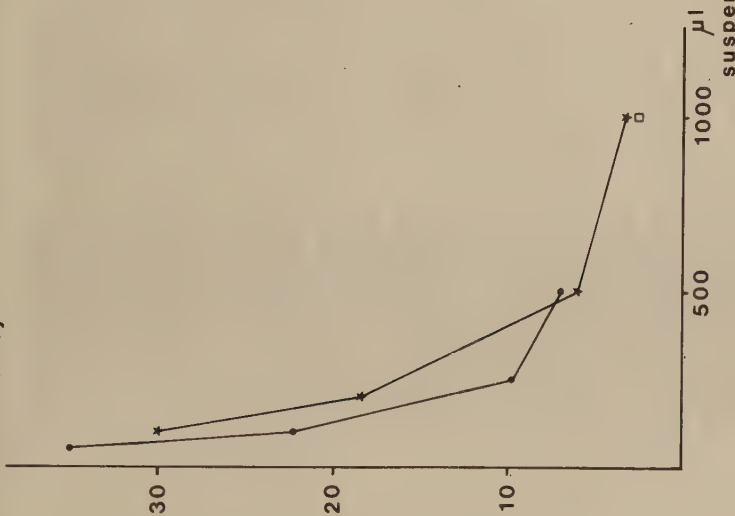


Fig. 2. The adsorptive capacity of starch-entrapped charcoal. Varying amounts of starch charcoal preparations containing 17 mg (★) or 34 mg (●) entrapped charcoal per ml microsphere suspension. Adsorption after addition of 6 mg ordinary charcoal (□). Initial radioactivity used was 30,000 cpm. After adsorption and sedimentation for 1 h, the supernatant was removed and counted.

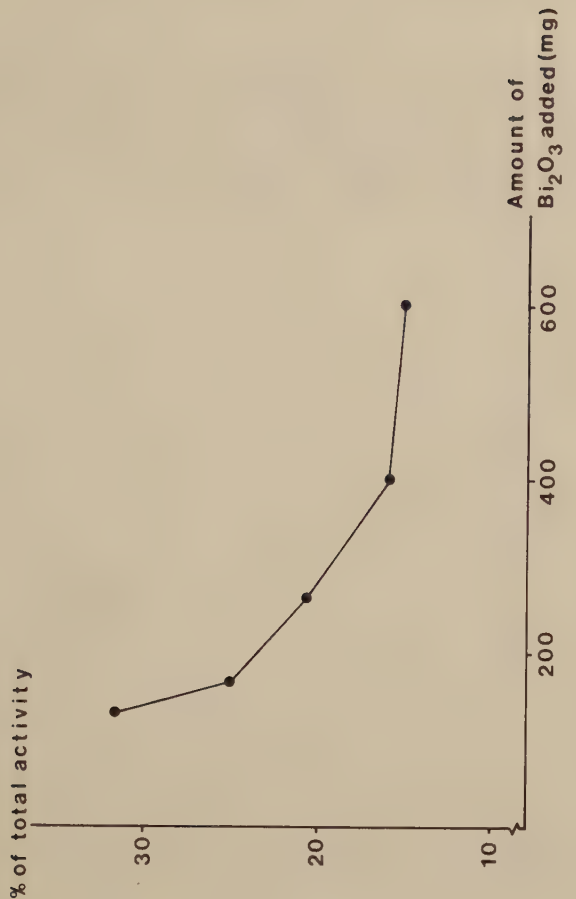


Fig. 3. Attenuating effect of varying amounts of entrapped bismuth oxide. The degree of attenuation is plotted as a function of the amount of entrapped bismuth oxide per ml microsphere suspension. 1 ml of starch spheres was mixed with 100 μl serum, 200 μl [^{125}I]T₃ (125 pg/ml) with ANS (1 mg/ml), 200 μl barbitol-0.25% BSA, pH 8.6. Sedimentation for 1 h prior to counting of the pellet.

% of total activity

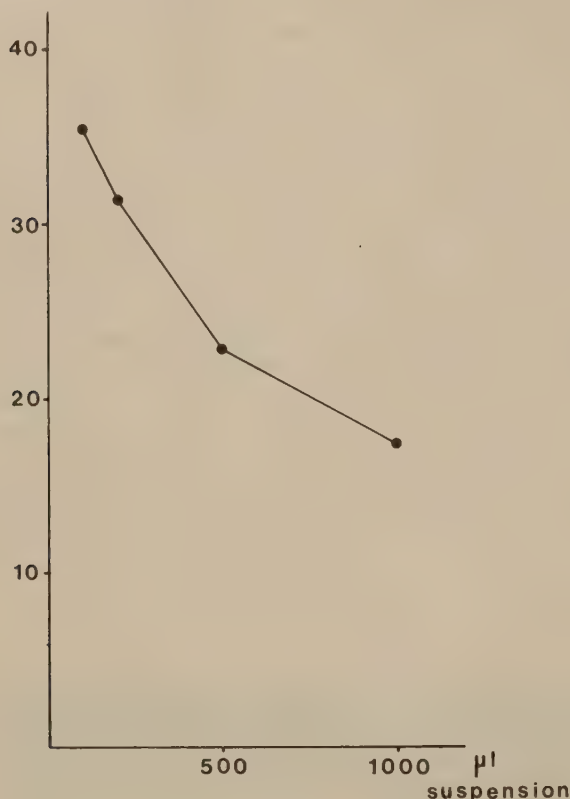


Fig. 4. Adsorption and attenuation by co-entrapped charcoal and bismuth oxide. Varying amounts of starch spheres containing bismuth oxide and charcoal (400 mg Bi_2O_3 and 17 mg of charcoal/ml microspheres suspension). Incubation conditions: 100 μl serum + 200 μl [^{125}I]T₃ (125 pg/ml, 1 mg ANS per ml) + 200 μl barbital-BSA + varying amounts of starch spheres. Barbital-BSA was added up to the total incubation volume of 1.5 ml. Sedimentation for 1 h before the supernatant was decanted and the non-quenched activity from the pellet was counted.

charcoal the entrapped retained 30–40% of its adsorptive capacity. In the following experiments 0.5 g of charcoal per 10 ml of beads was used (equivalent to 17 mg/ml suspension). To investigate the attenuation efficiency of entrapped bismuth oxide, beads containing 0.5 g of charcoal per 10 ml of beads and varying amounts of bismuth oxide were studied. It is clear from Fig. 3 that an increase in bismuth oxide content up to at least 12 g/10 ml of beads (equivalent to 400 mg bismuth oxide per ml suspension) was accompanied by a marked decrease in the activity in the pellet.

Owing to adsorption to the charcoal, the activity in the supernatant fell to 3–4%, whereas the level in the sediment was higher.

A typical standard curve for T_3 -assay is shown in Fig. 5. It shows a calibration curve, obtained after overnight incubation to allow the immunological reaction to take place prior to addition of the starch-bismuth oxide-

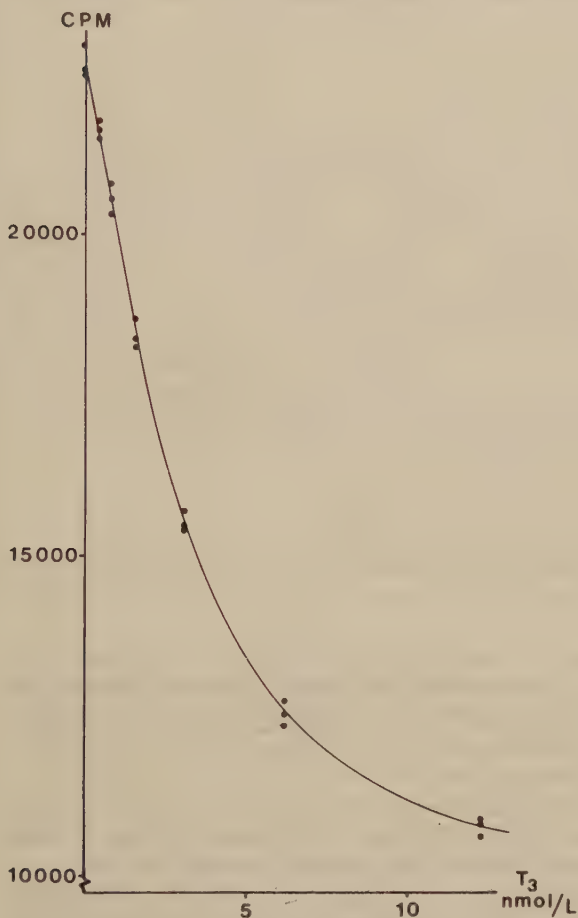


Fig. 5. Calibration curve for the radioimmunoassay of T_3 obtained from incubations containing 100 μ l serum + 200 μ l rabbit anti- T_3 (diluted 1:1000 with barbital buffer) + 200 μ l [125 I] T_3 (125 pg/ml, 1 mg ANS per ml). Incubation overnight before addition of 1 ml of starch spheres containing 400 mg bismuth oxide and 17 mg of charcoal per ml suspension. Sedimentation for 1 h prior to counting. Triplicates were measured.

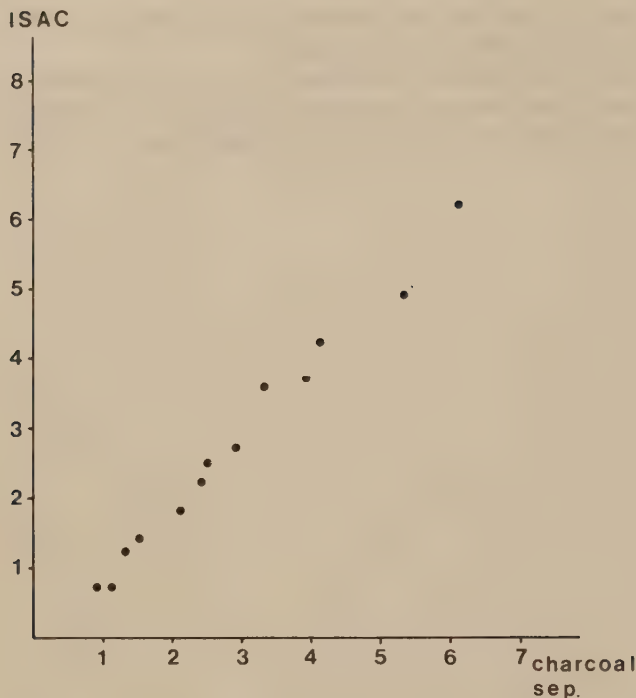


Fig. 6. The correlation between internal sample attenuator counting (ISAC) and a conventional radioimmunoassay of T_3 .

charcoal bead slurry. It can be seen from the standard curve that the concentration range assayed is well within the region of clinical interest. All assays were performed with a standardized lab. protocol since it was found that a slight drift in the counts occurred as a function of time. This phenomenon is receiving attention (to be published).

Patients' sera were assayed and the results were compared to those obtained by conventional procedures (Thorell and Larsson, 1978). A high correlation ($r=0.993$) was found between the analytical results (Fig. 6).

The reproducibility of this new technique was also tested. The within-day variation was 7.2%, calculated from triplicates of the calibration curve. Assays of pooled human serum containing 1.5 or 6.0 nmol T_3 /l on 5 different occasions showed a day-to-day variation of 10.3% and 7.8%, respectively.

In conclusion, we feel that this new approach to use an internal sample attenuator co-immobilized with the material binding either the free or the immunologically bound labelled antigen will prove useful in future radioimmunoassays, especially in automated assay systems.

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Combination of Solid-Phase Second Antibody and Internal Sample Attenuator Counting Techniques. Radioimmunoassay of Thyroid-Stimulating Hormone

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A method is described for the quantitation of free and bound antigen in a new radioimmunoassay which does not require centrifugation or decantation steps. The method involves coupling a second antibody to agarose spheres which also contain entrapped crystals of bismuth oxide (Bi_2O_3) in order to attenuate the γ -radiation from the bound fraction of the ^{125}I -labelled antigen. The system is designed so that the samples may be measured in a gamma counter 15 min after the spheres have been added.

This new method was compared to a conventional radioimmunoassay for the analysis of the thyroid-stimulating hormone (TSH). Data were obtained with the modified radioimmunoassay which showed both high sensitivity and precision and also had a good correlation ($r = 0.951$) with data obtained using a conventional method.

Key words: double antibody solid-phase radioimmunoassay – γ -radiation attenuator – thyroid-stimulating hormone

Introduction

The separation of bound from free antigen is important when performing radioimmunoassays. The design and performance of the separation step affects not only the results of the assay, but also the cost and work load involved. Of the many separation methods devised, the double antibody solid-phase (DASP) precipitation method combines versatility with a small 'misclassification error' (Wide and Porath, 1966). It has been used in many different radioimmunoassays and does not disturb the binding of the primary antigen to the antibody. To facilitate the handling of the separation step and the measurement of the radioactivity of the DASP method, we have combined it with the internal sample attenuator counting (ISAC) method. In ISAC, bismuth oxide is combined with the reagent and serves as an attenuator which

'hides' the radiation from either the free or the bound fraction within the test tube. It permits counting without physical separation of the fractions (Eriksson et al., 1981; Thorell, 1981).

This report describes the production of a solid-phase reagent which combines both the bismuth oxide attenuator and the second antibody into one particle by covalent binding of the second antibody to a matrix of agarose into which bismuth oxide is entrapped.

Materials

Agarose (type 1) and Tween 20 were obtained from Sigma, St. Louis, MO. The emulsifier Arlacel was a gift from Kemi-Intressen, Sundbyberg. Microcrystalline Cellulose Avicel® and bismuth oxide of 'reinst' grade were purchased from Merck, Darmstadt. Prior to use, the bismuth crystals were ground for 15 min to a maximal particle size of 5 μm using an Aeroplex-Spiralstrahlmühle 200 AS (Alpine AG, Augsburg). Orthophosphoric acid (about 100% H_3PO_4) came from BDH Chemicals Ltd., Poole and dextran T 70 from Pharmacia Fine Chemicals, Uppsala. Bovine serum albumin (BSA) fraction V was obtained from Amour Pharmaceutical Company, U.K. Goat anti-rabbit IgG immunoglobulin was produced by a 33% ammonium sulphate precipitation of goat serum. The precipitate was dialyzed against 0.05 mol/l phosphate buffer, pH 7.5. The concentration of IgG was adjusted to 10 g/l as determined from absorption at 280 nm ($\epsilon_{1\%} = 13.8$). ^{125}I -labelling of rabbit IgG was performed with the lactoperoxidase method (Thorell and Johansson, 1971). One hundred microlitres of rabbit IgG (3 g/l in 0.02 mol/l phosphate buffer, pH 6.6), isolated by ion exchange chromatography on DEAE Sephadex, were labelled with 10 μl Na^{125}I (3.7 GBq/ml, Radiochemical Centre, Amersham). The labelled rabbit IgG had a concentration of 50 $\mu\text{g/l}$ after separation of unbound ^{125}I . This corresponds to a final dilution of the serum of about 1 : 400,000, calculated from a normal serum concentration of 20 g IgG/l. For the binding studies native rabbit IgG was added to a concentration of 8 mg/l, which corresponds to a final serum dilution of 1 : 2500.

Methods

Preparation of microspheres

Eight hundred milligrams of agarose and 9 grams of Bi_2O_3 were mixed with 20 ml of 0.5 mol/l NaOH. The slurry was heated to 95°C and then poured into a 200 ml solution of ice-chilled toluene containing 20 g/l of the emulsifier Arlacel while being sonicated with an A 350 G type probe sonicator (350 W) (Ultrasonic, Yorks). Immediately afterwards, 200 μl of H_3PO_4 were added to the emulsion and 30 s later the sonication was stopped. The toluene mixture was then transferred to 1000 ml of acetone in which the spheres aggregated. After 5 min of sedimentation the acetone was aspirated off and the spheres were washed with another 500 ml of acetone on a

glass filter. The spheres were then suspended in 500 ml of water containing 1 g/l of dextran T 70 and sieved through a net with a pore size of 75 μm . After 30 min of sedimentation the water was decanted off and the spheres were resuspended in 500 ml of water and left to sediment for another 30 min. This washing procedure was repeated twice but in the last step the spheres were suspended in 0.1 mol/l Na_2HPO_4 . This preparation produced spheres with an approximate average size of 5 μm (range 1–20 μm) containing 450 g Bi_2O_3 /l. For the study of attenuator capacity spheres were also prepared containing 3, 6, 9 or 12 g Bi_2O_3 per 20 ml agarose giving a final concentration of 150, 300, 450 or 600 g entrapped Bi_2O_3 /l.

Covalent coupling of double antibodies to the microspheres

The coupling of immunoglobulin was performed using the CNBr method (Axen et al., 1967). The spheres were suspended in an equal volume of 0.1 mol/l Na_2HPO_4 and the pH was adjusted to 10.7 with 2 mol/l NaOH. CNBr was added (20 mg/ml) and the pH was maintained at 10.7 for 7 min by addition of 2 mol/l NaOH when required. The spheres were then collected by centrifugation for 30 s at $1000 \times g$ and washed twice with 0.1 mol/l Na_2HPO_4 , each time with 5 times the total volume of the spheres. After the washing steps the spheres were transferred to an equal volume of 0.1 mol/l Na_2HPO_4 buffer containing goat anti-rabbit IgG (5 g/l). The coupling proceeded with gentle shaking at 4°C overnight.

The next day the spheres were left to sediment and the unreacted protein was subsequently aspirated off before the spheres were resuspended in an equal volume of 1 mol/l ethanolamine and left for 2 h at room temperature to block any unreacted groups. The particles were then washed by decantation, twice with Na_2HPO_4 and once with 0.075 mol/l barbital buffer, pH 8.6, containing 2.5 g/l BSA (BBSA), to which 10 g/l dextran T 70 and 5 g/l Tween 20 had been added. Finally the spheres were suspended in an equal volume of BBSA, 10 g/l dextran T 70 and 5 g/l Tween 20.

Attenuation capacity of the spheres

One hundred microlitres of ^{125}I -labelled rabbit IgG diluted 1 : 1000 in BBSA, 100 μl human plasma and 100 μl of BBSA were mixed. Spheres suspended in BBSA with 1 g/l dextran T 70 containing various amounts of entrapped bismuth oxide were then added and the samples were mixed for 2 s. One hour later, after sedimentation of the beads, the samples were measured in an LKB Wallac 1280 Ultrogamma gamma counter (LKB, Bromma) using deep sample holders. Beads prepared by omitting the antibodies in the coupling step were used as a blank in this experiment.

Binding capacity of the spheres

Four hundred microlitres of a progressive dilution series of the spheres were added to a mixture containing 100 μl each of ^{125}I -labelled rabbit IgG, human plasma and BBSA. The dilutions were made with BBSA containing 1 g/l dextran T 70 and 12.5 g/l microcrystalline cellulose in order to get a proper pellet. After the samples were mixed for 2 s they were left standing on the bench for 1 h, centrifuged, and the supernatants removed and counted. For estimation of the total binding capacity of

the spheres, the tubes were incubated with gentle shaking for 2 h before their supernatants were removed and measured (Fig. 3).

Non-specific attenuation of ^{125}I -TSH from the bismuth spheres

Two hundred microlitres ^{125}I -TSH (15,000 cpm) were mixed with 300 μl BBSA or 200 μl BBSA and 100 μl serum. To the samples were added 200 μl of bismuth spheres suspended in BBSA or BBSA containing various amounts of dextran T 70 and Tween 20. One hour later the samples were measured in a gamma counter (Table I).

Assay of thyroid-stimulating hormone (TSH)

The primary incubation of the assay was performed according to a previously published method (Thorell and Larson, 1978). One hundred microlitres TSH standards (0, 2.5, 5.0, 10.0, 20, 40 and 80 mIU/l) in BBSA and 100 μl of rabbit anti-TSH serum diluted 1:6000 were incubated at 4°C. Four hours later 100 μl ^{125}I -labelled TSH was added to the samples and the incubation was continued overnight.

The next day, 200 μl of a suspension of bismuth spheres (equivalent to 45 mg Bi_2O_3 in BBSA containing 10 g/l dextran T 70 and 5 g/l Tween 20) were added and the samples were mixed (Vortexed) for 2 s. Thirty minutes later the samples were measured in a gamma counter and a calibration curve for TSH was constructed.

Results

Optimization of the attenuation and binding capacity of the double antibody spheres was performed with ^{125}I -labelled rabbit IgG as a tracer. The attenuation caused by different volumes of spheres and various amounts of entrapped Bi_2O_3 is shown in Fig. 1. Almost 90% of the precipitable activity was attenuated after addition of 200 μl of spheres containing 225 mg entrapped Bi_2O_3 /ml. Increasing the volume of spheres added or the content of Bi_2O_3 in the beads caused a higher non-specific attenuation. Volumes of the spheres smaller than 100 μl rapidly decreased the attenuation (Fig. 2).

The antibody binding capacity of the spheres was investigated by adding a progressive dilution series of the spheres to a fixed amount of ^{125}I -labelled rabbit IgG or to native IgG (8 mg/l) with ^{125}I -labelled IgG as a tracer (Fig. 3). Binding of ^{125}I -labelled IgG rapidly decreased with increasing dilution of the spheres and only the first and second dilutions had enough capacity to bind more than 90% of the labelled IgG. The binding was only modestly affected by the addition of native IgG. At higher dilutions, the addition of native IgG caused a certain decrease in binding. However, the binding capacity when the spheres sedimented in the tubes was only a small fraction of the total capacity obtained by shaking the samples for 2 h.

For further experiments, a 200 μl suspension of the spheres containing 225 g Bi_2O_3 /l was used.

The sedimentation and binding of the ^{125}I -labelled IgG was completed after 10

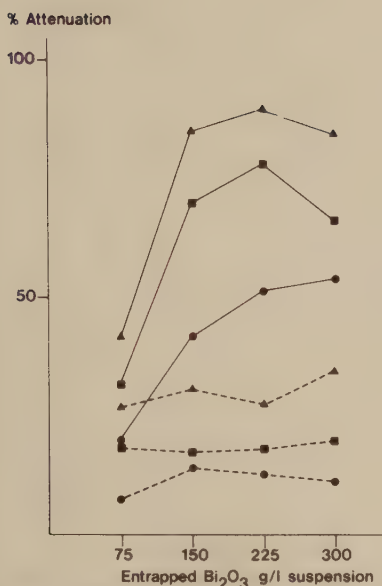


Fig. 1. Percentage of the total precipitable activity attenuated after addition of 3 different volumes of second antibody-agarose spheres containing various amounts of entrapped Bi_2O_3 (continuous line). Non-specific attenuation of the spheres was obtained when adding spheres without covalently coupled antibodies attached (discontinuous line). Activity after addition of 50 μl (●), 100 μl (■) or 200 μl (▲) of a suspension of the spheres. Experimental conditions were as described in Methods.

min and prolongation of the incubation up to 60 min did not alter the activity coming out of the tubes. Depending upon whether or not plasma was present in the samples, different amounts of ^{125}I -labelled TSH were adsorbed to the spheres. This

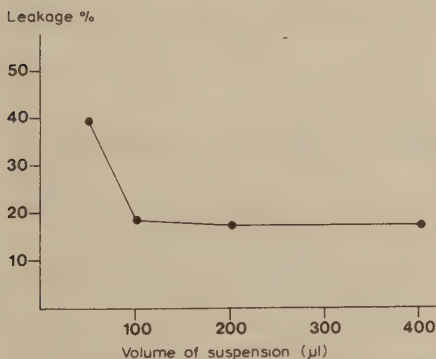


Fig. 2. Leakage of radioactivity from the pellet. Various amounts of a bismuth sphere suspension were added to ^{125}I -labelled rabbit IgG. After sedimentation the samples were centrifuged and the activity in the supernatants and pellets were counted separately. The activity in the supernatant was used to calculate the amount of activity bound to the pellet and the leakage of radioactivity from the pellet was expressed as % of the activity bound to the pellet.

Precipitable activity %

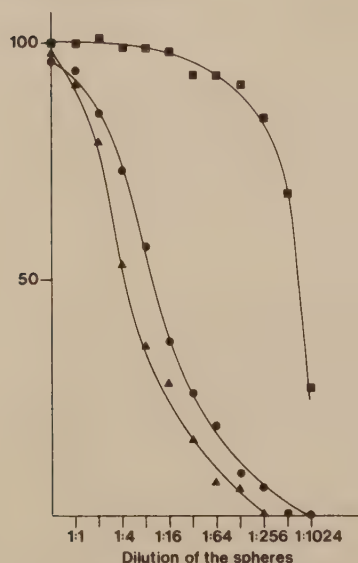


Fig. 3. Binding capacity of the bismuth spheres. Experimental conditions were as described in Methods. Sedimentation of the spheres (●). Bismuth spheres incubated with gentle shaking for 2 h (■). Sedimentation of bismuth spheres in samples containing ^{125}I -labelled rabbit IgG and 8 mg/l native rabbit IgG (▲).

non-specific adsorption was avoided by preincubating the spheres with dextran T 70 (10 g/l) and the detergent Tween 20 (20 g/l) (Table I).

The application of the spheres for the separation of bound and free antigen is exemplified with a typical assay of TSH (Fig. 4).

The sensitivity of the assay was 1.5 mIU TSH/l calculated from $2 \times \text{SD}$ for the

TABLE I

DATA ON NON-SPECIFIC ATTENUATION FROM MICROSPHERES WITH ENTRAPPED Bi_2O_3 AND COVALENT COUPLED DOUBLE ANTIBODY

Bismuth spheres suspended in:	Percentage of total activity	
	With serum	Without serum
BBSA	78.6	75.7
	79.6	77.1
BBSA containing 1 g/l dextran T 70	79.4	77.8
	81.0	78.6
BBSA containing 10 g/l dextran T 70 and 2 g/l Tween 20	79.1	79.0
	79.7	78.8
BBSA containing 10 g/l dextran T 70 and 5 g/l Tween 20	79.9	80.2
	78.6	79.8

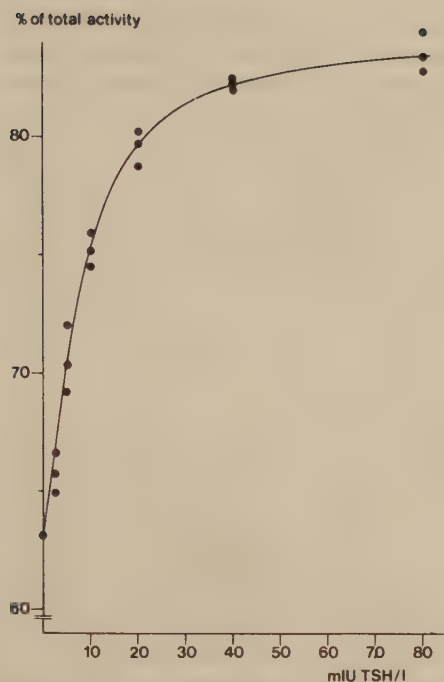


Fig. 4. Typical calibration curve for the thyroid-stimulating hormone (TSH) using the internal sample attenuator counting (ISAC) method. Experimental conditions were as described in Methods.

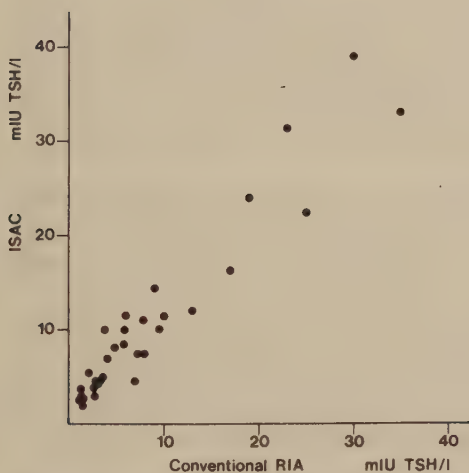


Fig. 5. Correlation between internal sample attenuator counting (ISAC) method and a conventional radioimmunoassay of TSH.

zero point of the calibration curve. The precision and reproducibility of the method was calculated from pooled human serum samples containing 7 or 30 mIU/l. Based on 20 measurements the intra-assay coefficient of variation (CV) was 9.2% and 11.0%, respectively. The inter-assay CV for the same sera as above measured on 20 different occasions, using 4 different preparations of bismuth spheres, was calculated to be 15.1% and 13.6%, respectively.

Patients' sera were assayed and the results were compared to those obtained by a conventional procedure (Thorell and Larson, 1978). Using 32 human serum TSH samples, the linear regression equation was $y = 1.06x + 1.92$ (Fig. 5) and the correlation coefficient was 0.951 ($P < 0.01$).

Discussion

This report demonstrates the feasibility of the combination of a double antibody separation method with an internal sample attenuator in radioimmunoassays. The attenuator was entrapped in an agarose matrix to which the specific binder could be covalently bound. With this combination, it was possible to achieve separation in a 1-step procedure. The precipitating capacity of this combined reagent was fully comparable with that obtained in conventional solid-phase double antibody precipitation methods in this laboratory. About 60% of the labelled material was precipitated.

The size of the agarose particles is critical. In initial experiments with spheres between 50–100 μm in size, the binding capacity was very low due to a high sedimentation rate of the spheres.

The marked increase in spherical binding capacity obtained if the samples were incubated with gentle shaking for 2 h shows that only a small portion of the coupled double antibody was used if the spheres were left to sediment. This indicates the possibility of increasing the binding efficiency by further modification of the spheres. The use of a more porous support may improve diffusion into the particles thus giving rapid access to a larger fraction of the antibody. Another alternative is the production of a very tight support in combination with smaller particle size to increase the effective binding capacity.

Addition of unlabelled rabbit IgG indicated that the spheres had enough binding capacity to bind all of the primary IgG in a radioimmunoassay when the final dilution of the antibodies was above 1:2500 (Fig. 3). Usually NaHCO_3 buffer is used when proteins are immobilized with the CNBr method (Axen et al., 1967). In this case, however, it was discovered that the entrapped bismuth oxide particles were changed to bismuth carbonate, which having a larger volume, ruptured the agarose spheres. The exposed bismuth carbonate also had the property of non-specifically adsorbing proteins, which made the spheres unsuitable for radioimmunoassays. For these reasons carbonate was avoided and the coupling of IgG was performed in 0.1 mol/l Na_2HPO_4 .

The assay of pooled serum samples containing known amounts of TSH and using the second antibody bismuth spheres gave falsely high estimates of TSH content.

This phenomenon was due to adsorption of free ^{125}I -labelled TSH to the spheres in the calibration measurements because the standard dilutions had been prepared in barbital buffer and not in serum. This non-specific adsorption of TSH to the spheres was prevented by coating the spheres with 10 g/l dextran T 70 and 5 g/l of the detergent Tween 20. After coating the particles, the same non-specific attenuation was obtained between samples containing serum or not (Table I) and the bismuth spheres showed a good correlation with a conventional double antibody precipitation method for the assay of TSH (Fig. 5, $y = 1.06x + 1.92$).

We conclude that the combination of solid-phase linked antibodies and internal sample attenuator counting techniques is a new alternative for distinguishing between free and bound antigen in a radioimmunoassay, thus simplifying the handling of the assay markedly.

Further development of this technique and immobilization of a primary anti-serum to the spheres may open up the possibility of performing equilibrium radioimmunoassays as well as kinetic radioimmunoassays.

Acknowledgements

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Methods of reducing the effect of spontaneous dissociation of antigen-antibody interactions in radioimmunoassays with special reference to internal sample attenuator counting (ISAC)

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A time dependent decrease of sample counts was observed in an adsorption radioimmunoassay with internal sample attenuator counting. The drift caused a bias in the estimate of the amount of antigen in the samples. The size of this deviation was dependent upon the length of time after the calibration curve was made that the samples were measured. This was essentially due to dissociation of the antigen-antibody complexes as the adsorber can act as a second receptor to the antigen. Addition of slowly sedimenting starch microspheres or starch particles inhibited the drift by forming a diffusion barrier on the attenuating pellet.

Key-words: attenuation; bismuth oxide; charcoal adsorption assay; drift of count rate; Gamma radiation; separation-induced immune complex dissociation

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The dextran coated charcoal adsorption method (DCC) has been widely used for the separation of bound and free radioactivity in radioimmunoassays [4]. The method is often applied in assays for small ligands like steroid and thyroid hormones [1, 7]. Advantages of the DCC method are instantaneous separation and inexpensive, reproducible reagents.

We have recently described the internal sample attenuator counting (ISAC), a new method

which significantly facilitates the separation and counting step of radioimmunoassays [8]. In one particular application we combined the DCC and the ISAC methods in assays for thyroid hormones [2].

It then became evident that the so called stripping effect could influence the results when long assay series were analysed, causing a drift in the count rate from the individual tube when they were measured over extended periods. The stripping problem is overcome by following rigid performance protocols, which standardize this effect so that it is identical in all test tubes. The

simplifications of the combined DCC-ISAC assay would be lost if this rigid protocol were followed.

The stripping effect is believed to be caused by binding of the antigen to the DCC. The binding interferes with the kinetics of antigen-antibody binding and results in some dissociation of antigen from the antigen-antibody complex [5]. However, the exact mechanisms have not been experimentally elucidated. Therefore, we investigated the stripping problem in some detail and devised a method which eliminates this problem.

MATERIALS

Carbohydrates: agarose (Medium Electroendosmosis) came from Marine Colloids Div., FMC Corp., Rockland, ME 04841, USA; starch, soluble AnalaR batch No. 2978430, BDH Chemicals Ltd., Poole, United Kingdom; corn starch, STA-Rx-1500, A.E. Staley Company, Illinois, USA; dextran, T70, Pharmacia Fine Chemicals AB, Uppsala, Sweden, and lactose, U.S.P. XX, AB Svenskt Mjölksocker, Mjölby, Sweden.

Charcoal, Aktivkohle zur Analyse, was purchased from Merck AG, Darmstadt, F.R.G. as was bismuth oxide of reagent grade, which was ground with an Aeroplex-Spiral-strahlmühle 200 As (Alpine AG, Augsburg, F.R.G.) to a maximal crystal dimension of 5 μm .

Detergents: Tween 20 was supplied by Sigma Chemical Co. St. Louis, MO, USA and GAFAC PE-510 by Svenska GAF, Stockholm, Sweden.

Triiodothyronine (T_3) came from Henning AG, Berlin, F.R.G. and ^{125}I -labelled T_3 was from New England Nuclear, Boston, MA 02118, USA.

Antisera against T_3 were raised in rabbits. T_3 conjugated to BSA with carbodiimide (Merck-Schuchardt, 80011 Hohenbrunn bei München, F.R.G.) [3] and Freund's complete adjuvant were used. Na^{125}I for the iodination of rabbit IgG using the lactoperoxidase method [6] was obtained from Amersham International Ltd., Amersham, Bucks, U.K.

The diluent for all reagents in the assays was 75 mmol/l barbital buffer, pH 8.6, containing 2.5 g of bovine serum albumin (BSA, Armour Pharmaceutical, Phoenix, AZ 85077, USA) per litre.

METHODS

Adsorber-attenuator reagent

Agarose matrix. Agarose particles were prepared as previously published [8]. Forty grams of bismuth oxide were mixed with 1.5 g of charcoal and 15 ml of hot agarose, 20 g/l in barbital buffer (without BSA). After 5 h in the refrigerator the gel formed was disintegrated and suspended in 100 ml barbital buffer containing 2.5 g BSA and 1.5 g of dextran T 70 per litre. The suspension was homogenized for 10 s and stored at 4°C.

Starch matrix. Microspheres of starch with entrapped Bi_2O_3 and charcoal were prepared as described earlier [2]. Twelve grams of Bi_2O_3 and 0.5 g of charcoal were mixed with 10 ml of water, containing 2.5 g Tween 20 per litre, and sonicated. Six grams of starch were added and the slurry was heated to 95°C. The slurry was rapidly poured into a stirred solution containing toluene and the emulsifier GAFAC PE-510 20 g per litre. The spheres formed were suspended in 200 ml of acetone and left to sediment for 5 min. The acetone was then aspirated off. This washing step was repeated four times. The spheres were then washed four times with 400 ml of water. Between the washings with water the spheres were left to sediment for 30 min. Finally, 1 g of wet spheres was suspended in 1 ml of barbital buffer containing 2.5 g BSA and 0.5 g dextran T 70 per litre and stored at 4°C.

Pure starch microspheres were prepared likewise, but by omitting the bismuth oxide and the charcoal. In the washing procedure with water the spheres were left to sediment for 1 h.

Radioimmunoassay of T_3

All assays and tests were performed in 11 $\times$ 55 mm polystyrene tubes with round bottoms. The assay was performed by incubating 100 μl of serum (or standards diluted in charcoal-treated serum), 200 μl of ^{125}I -labelled T_3 (25 pg) and 200 μl of rabbit antiserum against T_3 (diluted 1:1000) overnight at 4°C. The next day 1 ml of charcoal-bismuth agarose or starch microspheres was added. The samples were vortex-mixed for 2 s and the tubes were then left upright for 1 h before the radioactivity from the tubes was measured in an automated well-crystal gamma counter with a 39 mm deep well (LKB, Wallac ultragamma). The operational range of the calibration curve was 24–27% of the total

activity when shallow tubeholders which reached 5 mm above the bottom of the well were used. With deeper tubeholders that placed the tubes in the bottom of the well, the range was increased to 32–34% of the total activity.

Studies of the drift

The samples were repeatedly measured in the gamma counter and the observed decrease of counts was expressed as percentage of the value obtained 1 h after the addition of the adsorber-attenuator reagent. A linear regression analysis was used to calculate the decrease of activity as %/h.

The following materials were used as diffusion barriers in attempts to inhibit the drift:

1. 150 mg/ml lactose was added to the bismuth-charcoal agarose.
2. 0.1 g/ml of wet starch microspheres were added to the adsorber-attenuator reagent.
3. Varying amounts of solid corn starch or agarose particles were added to the adsorber-attenuator reagent.

RESULTS

Apparent drift

Calibration curves for the assay of triiodothyronine (T_3) with starch microspheres and with agarose particles, both containing charcoal and bismuth oxide, are shown in Figs 1 and 2 respectively. These show that after a time interval of 3 h the measured radioactivity had decreased.

The decrease of activity, expressed as change in percentage per unit time, was a linear phenomenon. The magnitude of the drift is given in Fig. 3 and Table I. The influence of the time interval on the apparent T_3 content is shown in Table II.

Characterization of the drift

The dependence of the drift on the presence of both the supernatant and the pellet was demonstrated in a series of measurements performed in tubes in which the particles had sedimented according to the standard assay procedure or in which the pellets and supernatants had been separated (Table III). The reduction after centrifugation is probably caused by packing of the pellet.

% of total activity

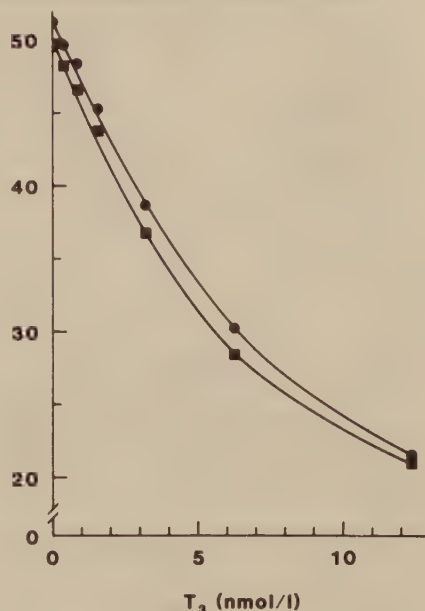


Fig. 1. Standard curve for triiodothyronine (T_3) assay with agarose particles containing Bi_2O_3 and charcoal (●). The same calibration curve measured 3 h later (■). Experimental details given in the text.

The importance of the contact area between the pellet and the supernatant is demonstrated by changing from standard tubes to wider tubes. Doubling the surface area between the pellet and the supernatant increased the drift from 0.4%/h to 0.8%/h. Otherwise the diameter of the tubes did not affect the range of the calibration curve.

Possible explanations for the drift could therefore be;

1. The antigen-antibody complexes are adsorbed to the sedimented pellet and attenuated.
2. After the adsorption of free antigen the equilibrium of the antigen-antibody binding reaction is changed, resulting in dissociation of antigen-antibody complexes in the solution. The antigen released from the immune complexes is adsorbed and attenuated by the pellet.

By substitution of ^{125}I - T_3 by ^{125}I -rabbit IgG no drift above the decay rate of ^{125}I was observed, even after a prolonged counting period (16 h).

% of total activity

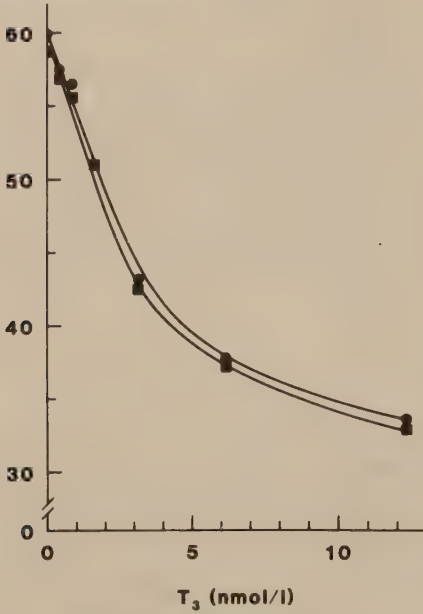


FIG. 2. Standard curve for triiodothyronine (T_3) assay with starch spheres containing Bi_2O_3 and charcoal (●). The same calibration curve measured 3 h later (■). Experimental details given in the text.

% of initial activity

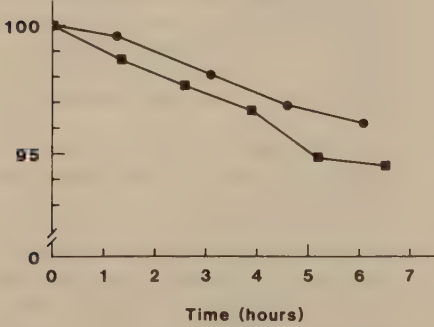


FIG. 3. Drift of O-standard (tube containing no unlabelled T_3). The tubes were measured repeatedly. The value measured after 1 h of sedimentation was set at 100%. Agarose particles containing entrapped Bi_2O_3 and charcoal (■). Starch spheres containing entrapped Bi_2O_3 and charcoal (●).

TABLE I. Decrease in activity within the calibration curve calculated over a period of 12 h

Amount of T_3 (nmol/l)	Decrease in activity as %/h of initial value*	
	Agarose matrix	Starch matrix
0	0.83±0.01	0.47±0.01
0.38	1.39±0.07	0.37±0.18
0.77	1.09±0.09	0.36±0.01
1.55	1.12±0.28	0.30±0.09
3.07	1.21±0.26	0.26±0.07
6.15	1.40±0.91	0.40±0.06
12.3	1.02±0.14	0.52±0.16

* Calculated from duplicates.

TABLE II. The effect of the drift on measured amounts of T_3 in a sample.

Time after measuring the calibration curve (min)	Measured amounts of T_3 (nmol/l)*			
	Agarose reagent		Starch reagent	
0	2.05	5.85	1.8	5.4
90			1.85	5.7
170	2.3	6.0	2.1	6.3
260			2.1	6.1
330	2.6	6.5		
540			2.4	6.7
780			3.0	7.5

* Mean from triplicates.

Further evidence for this hypothesis is a decrease in the drift observed after addition of 10-fold excess of T_3 antiserum to the suspension of the agarose particles (30% reduction of the drift).

Means of reducing the drift in the system

Accordingly the drift would be decreased by reducing the diffusion rate between the fluid and the solid phase. By the addition of lactose (150 mg/ml), to increase the viscosity, the drift was reduced to 0.3%/h. Starch microspheres without any entrapped charcoal or bismuth oxide were effective diffusion barriers. The drift was reduced from 0.9 to 0.16%/h if the starch microspheres were mixed with the agarose adsorber-attenuator reagent and from 0.4 to 0.15%/h if they were mixed with the starch adsorber-attenuator reagent. No reduction of

TABLE III. Drift in whole samples, separated pellets and supernatants with an agarose matrix over a period of 12 h*

Standard performance	Centrifuged samples (%/h)†		
	Supernatant and pellet remaining in the tube	Separated pellet	Separated supernatant
1.01±0.07	0.64±0.07	0.26±0.17	0.02±0.09‡

* mean of triplicates.

† after 1 h of sedimentation the samples were centrifuged. Pellets and supernatants were separated by decanting.

‡ decay rate of ^{125}I is 0.05%/h.

the drift was obtained if agarose particles were added to the adsorber-attenuator reagents.

The same effect as with the starch microspheres was obtained with soluble corn starch particles (Fig. 4). A calibration curve obtained with the agarose adsorber-attenuator reagent mixed with 10 g/l of soluble corn starch particles is seen in Fig. 5. The combination of the agarose adsorber-attenuator reagent and corn starch particles had an intra-assay variation of 8.6%, calculated from triplicates of the calibration points.

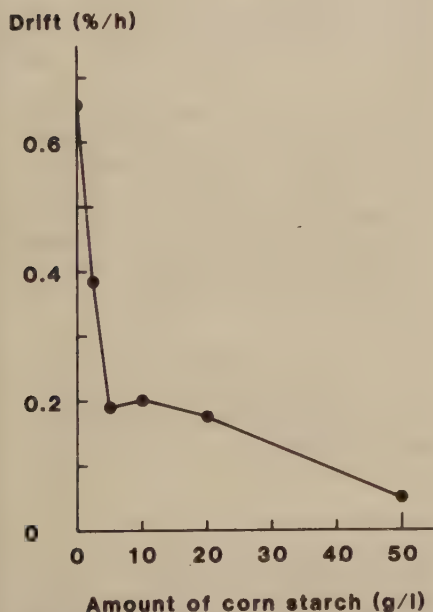


FIG. 4. Drift of O-standard in an agarose matrix particle assay after addition of varying amounts of corn starch to the adsorber-attenuator reagent.

DISCUSSION

During the development of a charcoal adsorption method for the ISAC technique, we found that the radioactivity emitted from the test tube decreased continuously during the time period in which the radioactivity was counted. The decrease was of the order of 1%/h, which is

% of total activity

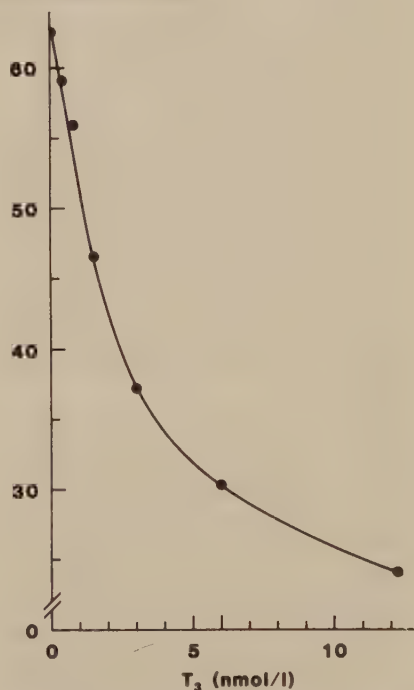


FIG. 5. Standard curve for triiodothyronine (T_3) assay with agarose adsorber-attenuator reagent mixed with 10 g/l of corn starch.

much higher than the decrease in the count rate caused by the radionuclide decay. Although the drift was small, it could cause a bias in the result of the assays if the interval between the incubation and the measurement of the first and last tubes of the assays differed by an extended period. The drift phenomenon observed here resembles the stripping effect of conventional DCC assays. Since the mechanisms behind this phenomenon were poorly understood the non-stability of the count rate in the assay was investigated.

The studies showed that the drift was not caused by events in either the supernatant or the sediment only, but that the presence of both phases was necessary. This excluded it being an effect caused by the redistribution of the radioactivity within either of the two phases by a gradual packing of the pellet which might have caused a gradual increase in the attenuation. Furthermore, it was shown that the rate of the drift was related to the area of the contact surface between the supernatant and the sediment. An exchange of radioactive material between the two phases was most likely. When the ^{125}I -antigen was substituted by ^{125}I -labelled antibody, no drift occurred. This indicates that the antigen-antibody complex as such was not involved in the drift. Furthermore, the addition of excess antibody to the supernatant, thereby reducing the dissociation rate of the antigen-antibody complex, decreased the drift. This observation also supports the hypothesis that the drift was caused by dissociation of the antigen-antibody complex, followed by diffusion of the antigen into the radiation attenuating pellet. Consequently, a restriction of the diffusion through the supernatant or into the pellet would reduce the drift.

Both the agarose and starch adsorber-attenuator reagents have enough capacity to adsorb all of the free label in the assay. The different reagents, however, differ in their attenuating capacity, although the same amounts of bismuth oxide are entrapped [2, 8].

Experiments also indicated that the drift was less prominent with a starch matrix (about 0.5%/h) than with an agarose matrix (about 1%/h). Since the agarose gel has larger pores and its carbohydrate skeleton constitutes a much smaller fraction of the particles (2% of the agarose matrix versus 38% of the starch matrix) the difference in attenuation and drift between the

reagents could be due to a restriction of antigen diffusion into the starch spheres, which would therefore mainly adsorb the antigen on their surfaces.

Overlaying the attenuating pellet with starch spheres containing no entrapped charcoal or bismuth oxide reduces the drift whereas agarose particles do not. This effect of starch spheres on the drift could therefore be due to impeded diffusion of released antigen from the antigen-antibody complexes to the attenuating pellet. A similar drift decreasing effect was obtained by restriction of the diffusion through the supernatant by the addition of lactose.

Finally, we demonstrated that the overlaying of plain starch particles on the pellet of bismuth oxide-containing particles virtually eliminated the drift. Quantitatively, there was no difference between the effect of retrograded starch microspheres or untreated starch.

The addition of starch did not affect the assay. The range of the calibration curve was the same (Fig. 5) and the precision of the assay was not changed. Calculated from the different calibration points, the intra-assay variation was 8.6% for the addition of corn starch to the agarose adsorber-attenuator reagent, 7.9% for the agarose adsorber-attenuator reagent alone and 7.2% for the starch adsorber-attenuator reagent alone [2, 8].

We conclude that the instability encountered in the adsorption type of separation system in radioimmunoassays is caused by dissociation of the antigen-antibody complex and adsorption of the released antigen to the adsorber. This effect could be minimized to the extent that it was not distinguishable from the decrease of the count rate caused by radionuclide decay.

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IV

LIPOSOME IMMUNE ASSAY (LIA). USE OF MEMBRANE ANTIGENS INSERTED INTO LABELED LIPID VESICLES AS TARGETS IN IMMUNE ASSAYS

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A new method is described for detection of membrane antigens and antibodies against such structures. By inserting partially purified rat transplantation antigen (RT-1) into iodine or fluorescein-labeled lipid vesicles, a precipitation of the liposomes is demonstrated by the use of a specific alloantiserum and a heterologous anti-rat IgG serum. Precipitation of liposomes carrying WF transplantation antigens could be detected at a final dilution of the alloantiserum of 1 : 3000, which is comparable to that obtained in a ⁵¹Cr-release assay. Furthermore, when unpurified membrane proteins from WF splenocytes were incorporated into labeled liposomes, an amount of RT-1 corresponding to 8000 cells could be detected.

INTRODUCTION

Membrane proteins can be extracted by two general methods (Shimada and Nathenson, 1967; Schwartz and Nathenson, 1971). Enzymatic cleavage by the use of papain gives at best the hydrophilic part of the membrane protein protruding from the membrane surface. However, an obvious disadvantage of this method is that cleavage may result in structural changes of the molecule. The other extraction principle involves a solubilization of the lipid component of the membrane by the use of detergent. As a result of such treatment the complete molecule of the membrane protein is obtained. If immunological methods are used in order to identify isolated membrane proteins the structural change or the presence of detergent (Quatiere et al., 1977) may influence the efficiency of the immune reaction. These problems can be avoided by insertion of the membrane proteins into artificial lipid vesicles.

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Incorporation of proteins from the major histocompatibility complex into liposomes has been described (Curman et al., 1978; Engelhard et al., 1978; Littman et al., 1979). It was shown that the majority of mouse H-2 and human HLA antigens expose their antigenic determinants externally when they are incorporated into liposomes. This offers the potential of using such liposomes as targets in immune assays. A technique has been described (Willoughby et al., 1978) whereby lysis of liposomes containing rat histocompatibility antigens (RT-1) by specific alloantisera was measured as the release of entrapped horseradish peroxidase.

In another system it has been shown that liposome lysis is easily detected by the use of spin-labeled markers instead of enzymes (Chan et al., 1978).

It is shown in the present investigation that when iodine or fluorescein-labeled lipids are introduced into the liposomal membrane, an immune precipitation of RT-1-carrying liposomes by specific alloantisera and a heterologous anti-rat IgG serum may be conveniently quantitated. This technique eliminates the problem of leakage that is seen when complement dependent lysis of antigen-carrying liposomes is used.

MATERIALS AND METHODS

Sephacrose CL-6B, DEAE Sephadex A-50 and Sephadex G-25 (PD-10 columns) were obtained from Pharmacia Fine Chemicals AB, Sweden. Phosphatidylcholine type V-E, phosphatidylserine, phosphatidylethanolamine type III, N-*t*-BOC-L-tyrosine, fluorescein isothiocyanate, N-hydroxysuccinimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, lactoperoxidase (E.C. No 1.11.1.7., 61 units/mg), deoxyribonuclease (E.C. No 3.1.4.5., DNAase, 1505 Kunitz units/mg) and 1-O-methyl- α -D-glucopyranoside were supplied from Sigma Chemical Co., U.S.A., and Minicon B15 was from Amicon, U.S.A. Sodium iodide (Na^{125}I) for protein iodination 3.7 GBq/ml was purchased from the Radiochemical Centre, Amersham, U.K. and Silica gel plates from Antec-Trägenplatten SL 254, Antec AC, 4127 Birsfelden, Switzerland. 0.1% ninhydrin and molybdatophosphoric acid spray reagent (Merck, Darmstadt, F.R.G.) were used for the detection of amino and phosphate groups on the thin layer chromatograms. Rat IgG was obtained from Cappel's Laboratories, U.S.A. All other reagents used were of analytical grade. Rats of the Wistar-Furth (WF) and Brown Norway (BN) inbred strains were used.

Preparation of lentil lectin

Lentil lectin was prepared by a slight modification of the method described by Howard et al. (1971). Four hundred grams of a commercial sample of lentil were allowed to swell overnight at 4°C in 2 liters of 0.9% NaCl, 0.2 mM MnCl_2 and 0.2 mM MgCl_2 . The lentils were then homogenized and the mixture was centrifuged at 13,000 $\times g$ for 20 min. The supernatant was further fractionated by ammonium sulfate precipitation. The material that precipitated between 30% and 80% saturation $(\text{NH}_4)_2\text{SO}_4$ was centri-

fuged and the pellet was dissolved in 200 ml 0.15 M sodium phosphate buffer, pH 7.5, and dialyzed against the same buffer. The dialyzate was applied on a Sephadex G-75 column (2 cm $\times$ 40 cm). After extensive washing of the column the lectin was eluted with 0.1 M α -methylglucopyranoside. The eluate was dialyzed against distilled water and lyophilized.

The coupling of lentil lectin to Sepharose CL-6B was made by the BrCN activation method (Axén et al., 1967). The activated Sepharose (0.07 g BrCN/g sucked Sepharose) was mixed with the protein (5 mg lentil lectin dissolved in 1 ml 0.1 M sodium bicarbonate per g Sepharose) and coupling proceeded under gentle shaking at 4°C overnight. Blocking of the unreacted groups was done by incubation for 1 h with 0.5 M ethanolamine, pH 8.5, at room temperature.

Partial purification of histocompatibility antigens RT-1 from WF rats

One hundred grams of spleen tissue from WF rats were homogenized in 300 ml ice-cold phosphate-buffered saline (PBS), pH 7.4, with an Omni Mixer (setting 6 for 1 min). The homogenate was centrifuged at 1000 $\times g$ for 10 min. The supernatant was collected and the pellet was suspended in 100 ml PBS and recentrifuged.

The two supernatants were pooled and centrifuged at 100,000 $\times g$ for 1 h. The pellet was washed twice with 100 ml PBS, solubilized in 50 ml 20 mM Tris-HCl, pH 8.1, containing 1% (w/v) sodium deoxycholate (DOC), stirred for 10 min at 4°C and centrifuged at 100,000 $\times g$ for 1 h. The solubilized membrane proteins were applied on a lentil lectin Sepharose CL-6B column, equilibrated with 1% DOC (w/v) in 10 mM Tris-HCl, pH 8.1. After extensive washing in the same buffer the glycoproteins were eluted with 0.1 M α -methyl-glucopyranoside in 1% DOC, 10 mM Tris-HCl, pH 8.1. The eluate containing transplantation antigens was concentrated to approximately 1 mg/ml in a Minicon-B15. Protein determination was carried out according to Lowry et al. (1951).

Conjugation of phospholipids and N-t-BOC-L-tyrosine

Phospholipids and N-t-BOC-L-tyrosine were conjugated by use of the carbodiimide method (Cuatrecasas and Parikh, 1972). N-t-BOC-L-tyrosine (0.1 mmol) was dissolved in 1 ml dioxane. Solid N-hydroxysuccinimide (0.1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.1 mmol) were added to the tyrosine solution and this was stirred for 70 min at room temperature. The activated N-t-BOC-L-tyrosine was transferred to a glass tube containing 10 mg of phosphatidylserine ($\sim 13 \mu\text{mol}$) which, in order to remove the chloroform : methanol solvent, had been dried under nitrogen into a thin film on the glass tube. The coupling reaction was continued by stirring for 12 h at 4°C. The dioxane was evaporated and the conjugate was solubilized in 2 ml chloroform. In order to remove undissolved material (appearing

as a flotat) the solution was centrifuged three times at $3000 \times g$ for 10 min at room temperature. Before each centrifugation the chloroform solution was placed in a freezer to decrease the solubility of the floating material. After each centrifugation undissolved material appearing as white floating material on the top of the chloroform was discarded. The volume was adjusted to 2 ml with chloroform and stored at -20°C .

In order to investigate the composition of the flotat, all the undissolved material was collected, washed once with 2 ml chloroform and dried under a stream of nitrogen. The flotat was then dissolved in dioxane and assayed with HPLC using a C_{18} -silica column and as eluent dioxane : water (1 : 1 (v/v)). This procedure showed that 70% of the modified N-*t*-BOC-L-tyrosine appeared in the flotat. Minor amounts of N-hydroxysuccinimide were also detected.

Five mg phosphatidylethanolamine ($\sim 7 \mu\text{mol}$) dissolved in chloroform : methanol were dried under nitrogen into a thin film on a glass tube and dissolved in 0.5 ml dioxane. The phospholipid solution was transferred to a test tube containing N-*t*-BOC-L-tyrosine (10 μmol), N-hydroxysuccinimide (50 μmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (50 μmol). The solution was stirred for 2 h at room temperature and then overnight at 4°C . The next day the dioxane was evaporated under nitrogen and the formed phospholipid-tyrosine conjugate was dissolved in 2 ml chloroform : methanol (95 : 5 (v/v)) and stored at -20°C .

Thin layer chromatography (silica gel plate using a mobile phase of chloroform-acetone-methanol-acetic acid-water 3 : 4 : 1 : 1 : 0.5 (v/v)) revealed after spraying with 0.1% ninhydrin solution that free amino groups of the phosphatidylserine were still present after the conjugation step. No indication of free amino groups was seen after the phosphatidylethanolamine conjugation. When examined in UV-light, thin layer chromatograms of the phosphatidylserine conjugation mixture also showed a spot with the same R_f value as that of N-*t*-BOC-L-tyrosine. When the thin layer chromatograms of the phospholipid conjugates were tested for phosphate with 'Molybdatophosphorsäure', blue spots appeared that were not present on plates with unreacted phospholipids. Chromatograms of phosphatidylserine conjugate showed a phosphate-containing spot with the same R_f value (0.9–1.0) as that of N-*t*-BOC-L-tyrosine (when examined in UV-light), whereas native phosphatidylserine had R_f values of 0–0.47. Chromatograms of the phosphatidylethanolamine conjugate showed a main spot with an R_f value of 0.76. Minor impurities with R_f values 0.54 and 0.62 were also observed. No spots with the same R_f value as native phosphatidylethanolamine were observed (R_f 0.34).

To quantitate the amount of unreacted phosphatidylserine the conjugation mixture was separated with HPLC using a C_{18} -silica column. When dioxane : water (4 : 1 (v/v)) was used as the mobile phase, a retarded fraction with absorbance at 276 nm was collected. This fraction was assayed for phosphate with ammonium molybdate (Fishe and SubbaRow, 1925) and

was found to contain 24% of the total phosphate of the conjugation mixture.

¹²⁵I-labeling of phospholipid-tyrosine conjugate

The ¹²⁵I-labeling of phospholipid-tyrosine conjugate was done according to the lactoperoxidase method (Thorell and Johansson, 1971). Two hundred μ l phospholipid-tyrosine conjugate were dried under nitrogen into a thin film on a glass tube. The phosphatidylserine-tyrosine conjugate was dissolved in 0.5 ml, and the phosphatidylethanolamine-tyrosine conjugate in 0.2 ml 1% DOC, 10 mM Tris-HCl, pH 8.1. One hundred μ l of the dissolved phospholipid-tyrosine conjugate were added to 10 μ l Na¹²⁵I (3.7 GBq/ml). Two μ l lactoperoxidase (2 mg/ml) and 10 μ l H₂O₂ (0.003% v/v) were added under magnetic stirring. After 30 sec the iodination was stopped by adding 0.4 ml 1% DOC, 10 mM Tris-HCl, pH 8.1. The unbound ¹²⁵I was separated from the conjugate through a Sephadex G-25 column (PD-10) equilibrated with 1% DOC, 10 mM Tris-HCl, pH 8.1. The fraction eluted in the void volume from the column was collected and stored at 4°C. The yield from the iodination of the phosphatidylserine-N-*t*-BOC-L-tyrosine was about 10% and of the phosphatidylethanolamine-N-*t*-BOC-L-tyrosine about 40%.

Conjugation of fluorescein to phosphatidylserine

Five mg phosphatidylserine dissolved in chloroform : methanol (95 : 5) were dried under nitrogen into a thin film on a glass tube and then dissolved in 500 μ l 0.5 M sodium carbonate buffer, pH 9.5, containing 2% DOC (w/v). Two hundred μ l fluorescein isothiocyanate (10 mg/ml) dissolved in the same buffer, were added to the solution and were allowed to react for 90 min at 30°C. The reaction mixture was then applied on a Sephadex G-10 column (20 cm $\times$ 1 cm) which had been equilibrated with the same buffer. The fluorescent material that did not stick to the column was recovered and stored at 4°C.

Liposome preparation

Varying amounts of phosphatidylcholine, ¹²⁵I-labeled phospholipid-tyrosine conjugate and glycoprotein isolated by affinity chromatography on lentil lectin Sepharose CL-6B were mixed. All these components were dissolved in 10 mM Tris-HCl, pH 8.1, 1% DOC and applied on a Sephadex G-25 column (PD-10), previously equilibrated with a buffer (10 mM Tris-HCl, pH 8.1, 150 mM NaCl) containing no detergent. Fractions of 1 ml were collected and measured in a gamma counter. The material found in the void volume which contained glycoproteins inserted into ¹²⁵I-labeled liposomes was collected. Fluorescein-labeled liposomes were prepared in the same manner as ¹²⁵I-labeled liposomes except that the radioactive lipid was substituted by a fluorescent phospholipid conjugate.

Immunization procedures

Rats of the WF and BN strains were bred by strict sibling mating. Adult animals of the BN strain were immunized by three biweekly injections i.p. of 2×10^7 allogeneic spleen and lymph node cells. Anti-rat IgG was raised in rabbits by two bimonthly intramuscular injections of 1 mg rat IgG purified by DEAE-Sephadex chromatography.

Liposome immune assay (LIA)

^{125}I -labeled liposomes containing WF spleen tissue glycoproteins isolated by affinity chromatography on lentil lectin Sepharose CL-6B were incubated with serum from untreated BN rats or from BN rats immunized against transplantation antigens from WF rats. After the incubation a rabbit anti-rat IgG serum was added and the formed immune precipitate was carefully layered over a 200 μl 0.5 M sucrose cushion in 10 mM Tris-HCl, pH 8.1, in Spinco microtest tubes (500 μl) and centrifuged at $2800 \times g$ at room temperature for 10 min. After centrifugation the tubes were frozen at -80°C and the bottom tips of the tubes containing the precipitate were cut off and radioactivity was measured in a gamma counter. The precipitation of fluorescein-labeled liposomes was carried out in the same way as ^{125}I -labeled liposomes. After cutting the tip off the tube, the precipitate was suspended in the sucrose content of the tip and transferred to a test tube. The empty tip was washed by filling it with 4% sodium dodecyl sulfate in 10% mercaptoethanol (approximately 50 μl) and this volume was added to the suspended precipitate. The mixture was boiled for 5 min and 2.5 ml 1% DOC, 10 mM Tris-HCl, pH 8.1, were added and the solution was measured in a spectrofluorometer (excitation wavelength: 490 nm; emitting wavelength: 515 nm).

RESULTS

When liposomes containing histocompatibility antigens are incubated with an appropriate antiserum and subsequently exposed to a secondary antibody it is to be expected that co-precipitation of liposomes should occur. Quantitation of this precipitation should be readily achieved on the basis of the introduced phospholipid conjugate.

When fluorescein-labeled liposomes containing WF transplantation antigens were incubated with BN anti-WF serum and then with a rabbit anti-rat IgG serum, fluorescein-labeled precipitate could be identified by fluorescence microscopy of the suspended immune precipitate (Fig. 1). The specificity of the immune precipitate is illustrated in Fig. 2, showing a much higher amount of precipitated fluorescein-labeled liposomes when BN anti-WF serum was used compared to normal BN serum. When ^{125}I -labeled phospholipid conjugate was used instead of fluorescein-labeled lipid it was possible to detect a pellet containing radioactive liposomes after co-precipitation.

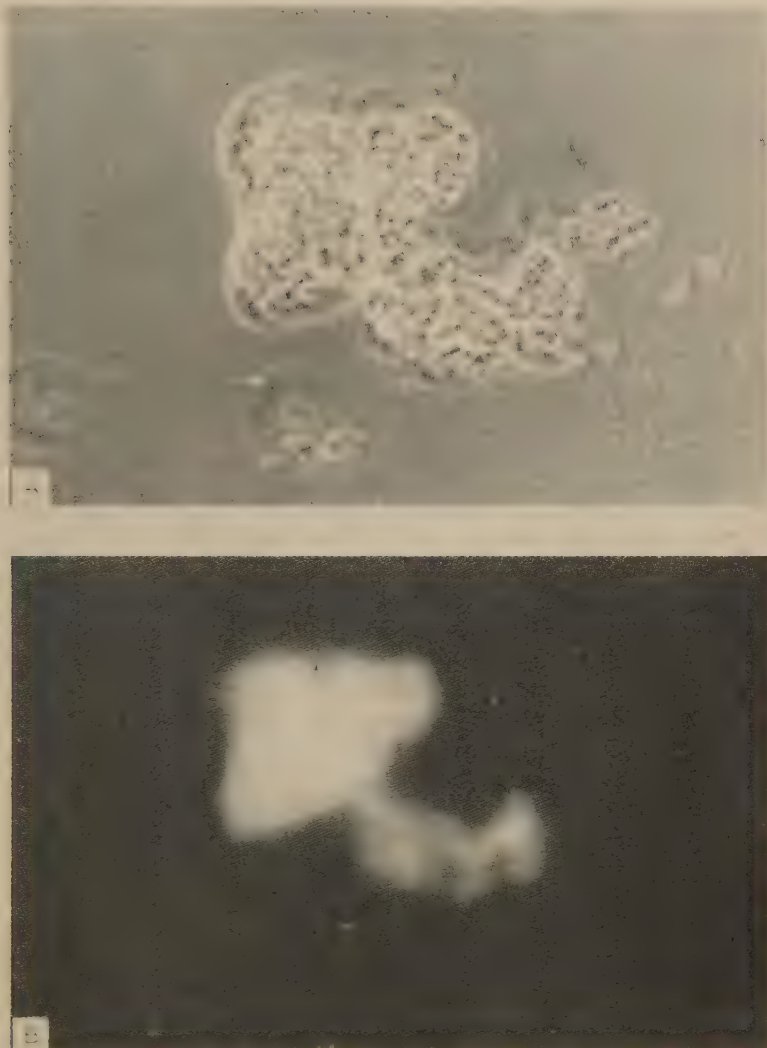


Fig. 1. Micrographs of a sample of immunoprecipitated fluorescein-labeled, liposomes containing RT-1. Seventy-five μ l liposome suspension containing 100 μ g phosphatidylcholine, 5 μ g fluorescein-labeled phosphatidylserine and 3 μ g glycoproteins from spleen tissue of WF rats were incubated with 2 μ l BN anti-WF serum for 1 h at 20°C and then with 25 μ l rabbit anti-rat IgG serum for 1 h. A Leitz Dialux microscope fitted with a 12 V, 100 W tungsten-halogen lamp was used (A). For detection of fluorescence, the same microscope supplemented with Ploemopak 3 filterblock, BG 38 and PK 500 excitation filters and K515 and S525 suppression filters was used (B). Magnification $\times 325$.

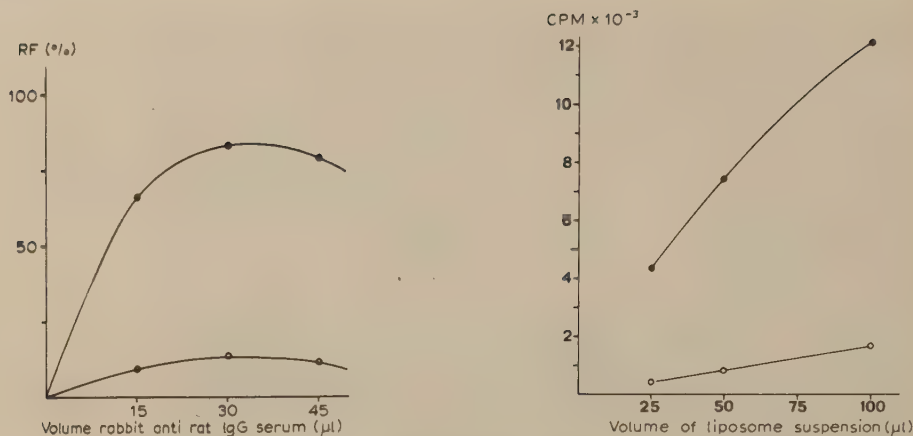


Fig. 2. Relative fluorescence (RF) of immunoprecipitate of fluorescein-labeled liposomes containing RT-1. For each incubation, 50 μ l liposome suspension containing 70 μ g phosphatidylcholine, 3 μ g fluorescein-labeled phosphatidylserine and 2 μ g glycoprotein from spleen tissue of WF rats were used. The liposomes were incubated at 20°C for 1 h with 2 μ l BN anti-WF serum (●) or normal BN serum (○) then for 1 h with varying amounts of rabbits anti-rat IgG serum. A relative fluorescence of 100% corresponds to the fluorescence of 5 μ g phospholipid mixture.

Fig. 3. Radioactivity of the immunoprecipitate from varying amounts of 125 I-labeled liposomes containing RT-1. Varying amounts of liposomes (1.3 mg phosphatidylcholine, 2×10^6 cpm 125 I-labeled phosphatidylserine conjugate and 45 μ g glycoprotein from spleen tissue of WF rats per ml liposome suspension) to a total volume of 100 μ l were incubated for 1 h at 20°C with 2 μ l BN anti-WF serum (●) or 2 μ l normal BN serum (○). Twenty-five μ l rabbit anti-rat IgG serum was then added and incubated for 1 h at 20°C.

An almost linear increase of the radioactivity recovered in the precipitate was observed when increasing amounts of 125 I-labeled liposomes were incubated with 2 μ l serum both when BN anti-WF serum and normal BN serum were used as the first antibody (Fig. 3). Approximately 10 times higher values were obtained when BN anti-WF serum was used compared to normal BN serum. The figure also shows that when 100 μ l liposomes were used, which corresponds to 330 μ g phosphatidylcholine and 12 μ g glycoproteins obtained from a lentil lectin affinity column, the amount of antiserum is not limiting up to 100 μ l liposome suspension.

The optimal amounts of primary and secondary antibodies were investigated by incubating liposomes containing antigens and 125 I-labeled phospholipid conjugate incorporated into the lipid bilayer, with 0.5, 1.0 and 2.0 μ l primary antiserum and varying amounts of rabbit anti-rat IgG serum (Fig. 4). The radioactivity of the immune precipitates increased with increasing amounts of primary antiserum. The activity found in the pellet after varia-

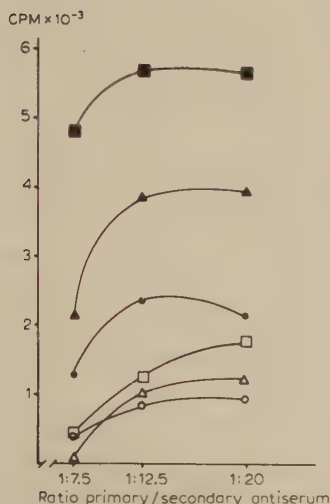


Fig. 4. Radioactivity of the immunoprecipitate from ^{125}I -labeled liposomes containing RT-1. For each incubation, 100 μl liposome suspension containing 70 μg phosphatidylcholine, 1×10^5 cpm ^{125}I -labeled phosphatidylserine conjugate and 1.4 μg glycoprotein from spleen tissue of WF rats were used. The liposomes were incubated for 1 h at 20°C with 2 μl (■), 1 μl (▲) and 0.5 μl (●) BN anti-WF serum, or 2 μl (□), 1 μl (△) and 0.5 μl (○) normal BN serum and then for 1 h with rabbit anti-rat IgG serum. The secondary antiserum was added to give primary/secondary antiserum ratios of 1 : 7.5, 1 : 12.5 and 1 : 20 and incubated at 20°C for 1 h.

tion of the secondary antibody showed a maximum when the ratio primary : secondary antibody was 1 : 12.5.

The time and temperature dependence of the liposome immune assay was investigated by incubating both the primary and secondary antibody for varying periods of time at 20°C and at 4°C . The reaction with antigen-carrying liposomes was completed after 4 h of incubation with BN anti-WF serum, both when the incubation was made at 20°C and at 4°C (Fig. 5). When the reaction was continued for another 16 h, a slight decrease of the radioactivity was seen if the incubation was carried out at room temperature, whereas no such decrease was seen at 4°C . Constant values of radioactivity were found in the pellet when normal BN serum was used as primary antibody and incubated for varying periods of time with antigen-carrying liposomes. The immune precipitation of antibody-coated RT-1 containing liposomes was completed after 1 h of incubation with a rabbit anti-rat IgG serum (Fig. 6). A decrease of the radioactivity was found in the pellet when incubation with the secondary antibody was carried out for 4 h. In the case of control BN serum used as primary serum the radioactivity of the pellet did not change on prolonged incubation with the secondary antiserum.

The detection limit of the liposome immune assay regarding antibodies

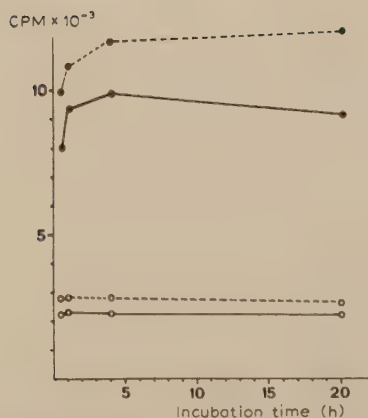


Fig. 5. Radioactivity of immunoprecipitates of ^{125}I -labeled liposomes containing RT-1. For each incubation, 50 μl liposome suspension containing 60 μg phosphatidylcholine, 1×10^5 cpm ^{125}I -labeled phosphatidylethanolamine conjugate and 6 μg glycoproteins from spleen tissue of WF rats were used. The liposomes were incubated at 4°C (— · — · —) and 20°C (—) for varying periods of time with 2 μl BN anti-WF serum (●) and 2 μl normal BN serum (○) and then with 25 μl rabbit anti-rat IgG serum for 1 h at the same temperatures.

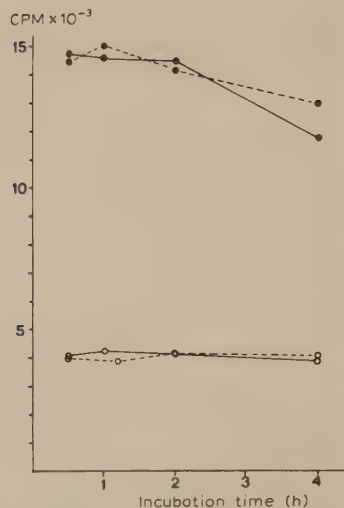


Fig. 6. Radioactivity of immunoprecipitates of ^{125}I -labeled liposomes containing RT-1. For each incubation 50 μl liposome suspension containing 53 μg phosphatidylcholine, 1×10^5 cpm ^{125}I -labeled phosphatidylethanolamine conjugate and 10 μg glycoprotein from spleen tissue of WF rats were used. The liposomes were incubated at 4°C (— · — · —) and 20°C (—) for 1 h with 2 μl BN anti-WF serum (●) or 2 μl normal BN serum (○) and then with 25 μl rabbit anti-rat IgG serum for varying periods of time at the same temperatures.

against a membrane antigen was investigated. One hundred μl of ^{125}I -labeled liposome suspension containing 330 μg phosphatidylcholine and 12 μg glycoprotein from an eluate of a lentil lectin affinity column were incubated with various dilutions of BN anti-WF serum. Specific antibodies were detected in a final dilution titer of 1 : 3000 (Fig. 7). The same results were obtained when antigen-carrying liposomes were incubated with BN anti-WF serum for 1 h at room temperature as for 12 h at 4°C.

DISCUSSION

When a conventional radioimmune assay is used, the antigen is often labeled with iodine. This iodination might be harmful, as the introduction of iodine might change the physical properties of the molecule and thereby

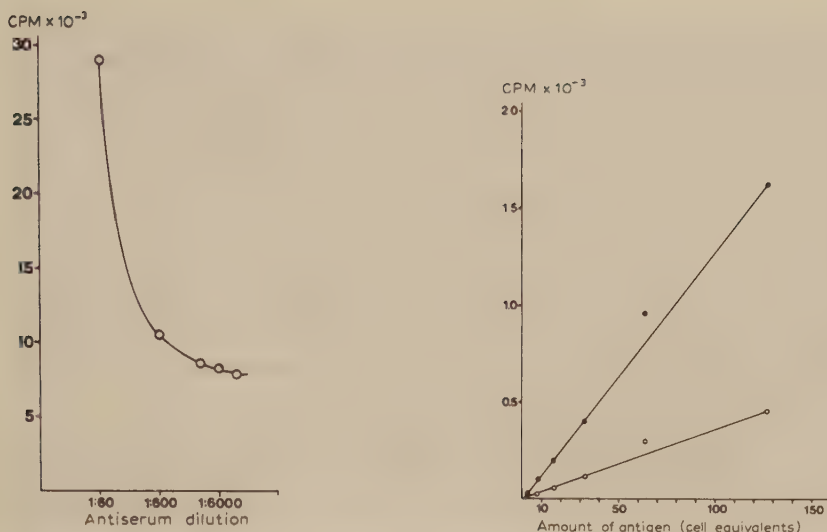


Fig. 7. Radioactivity of the immunoprecipitate from ^{125}I -labeled liposomes containing RT-1 incubated with varying dilutions of BN anti-WF serum. For each incubation, 100 μl liposome suspension containing 100 μg phosphatidylcholine, 1×10^5 cpm labeled phosphatidylethanolamine conjugate and 19 μg glycoprotein from spleen tissue of WF rats were used. The liposomes were incubated with 20 μl of varying dilution of BN anti-WF serum for 16 h at 4°C and precipitated with 25 μl rabbit anti-rat IgG serum for 1 h. Prior to the addition of the secondary antiserum, 2 μl BN normal serum was added to all of the samples except the lowest dilution of BN anti-WF serum which corresponds to 2 μl serum.

Fig. 8. Radioactivity of immunoprecipitate of ^{125}I -labeled liposomes containing RT-1. 140×10^6 splenocytes were washed twice with PBS and then solubilized in 1.4 ml 20 mM Tris-HCl, pH 8.1, 1% DOC in the presence of 100 $\mu\text{g}/\text{ml}$ DNase. After 10 min the solubilized cells were centrifuged at $10,000 \times g$ for 10 min and the supernatant was taken. Liposomes containing RT-1 were made from 200 μl of the solubilized material, 0.5 mg phosphatidylcholine and 0.5×10^6 cpm ^{125}I -labeled phosphatidylethanolamine conjugate. Various dilutions of liposomes were incubated with 2 μl BN anti-WF serum (●) or 2 μl normal BN serum (○) for 1 h at 20°C and then for 1 h with 25 μl rabbit anti-rat IgG serum. The amount of antigen inserted into liposomes is expressed as cell equivalents, which is the amount of protein corresponding to the given number of cells.

alter its antigenicity (Brunfeldt et al., 1968). Another limiting factor is that the iodination requires a specific amino acid which might be absent in some cases. An assay based on immune precipitation of membrane proteins which are inserted into labeled lipid vesicles circumvents this type of problem. Furthermore, the use of labeled phospholipids and native membrane proteins in the same liposome makes it possible to use a much heavier labeling than if only the protein part was to be modified. This fact contributes to an increased sensitivity in the assay.

In order to label the lipid with radioactive iodine a conjugation of tyrosine and the lipid is performed. Thin layer chromatography of the reaction mixture of phosphatidylserine and *N*-*t*-BOC-L-tyrosine revealed that even at 10 times excess of amino acid, free amino groups of the lipid could be detected. The low yield (10%) from the iodination of the phosphatidylserine conjugate can be explained by the presence of unreacted *N*-*t*-BOC-L-tyrosine which also becomes iodinated. The phosphatidylethanolamine, however, contains no carboxyl groups which makes it possible to activate the *N*-*t*-BOC-L-tyrosine in the presence of the phospholipid. This results in a more efficient ^{125}I -labeling of the lipid conjugate. Another way to label the phospholipid is to conjugate phosphatidylserine with fluorescein isothiocyanate and the use of that conjugate could serve as a suitable alternative to the iodinated lipid in the liposome immune assay.

In order to optimize the conditions for the liposome immune assay, the ratio between primary and secondary antisera was varied. Figs. 3 and 7 show that the best RT-1 liposome precipitating effect was obtained when 2 μl primary and 25 μl secondary antiserum were used. These volumes were used for further experiments.

The immune reaction between BN anti-WF serum and WF RT-1 carrying liposomes reached an equilibrium after 4 h of incubation and remained constant for at least 20 h at 4°C. A slight decrease of the radioactivity was observed after 22 h of incubation at 20°C. The reaction between primary and secondary antiserum was completed within 1 h. A clear decrease in radioactivity was observed when the reaction was allowed to continue for a longer period of time. The time-dependent decrease of radioactivity might be explained by effects similar to shedding which have been observed on intact cells (Unanue and Karnovsky, 1973).

The liposome immune assay is a sensitive method for detection of specific antibodies against membrane antigens indicated by the fact that specific immune precipitation of RT-1-carrying liposomes by a BN anti-WF serum was demonstrated at a final serum dilution of 1 : 3000. This is closely similar to the titer determined by complement-dependent lysis of ^{51}Cr -labeled WF cells (unpublished result). The liposome immune assay is also sensitive with respect to detection of antigen illustrated by the fact that a specific precipitation of liposomes containing proteins from 8000 spleen cells can be detected when a BN anti-WF serum and a rabbit anti-rat IgG is used (Fig. 8). It has been demonstrated that there are three times more β_2 microglobulin molecules than HLA molecules in human lymphocytes (Dorval et al., 1977). If this ratio is valid for rat lymphocytes as well, the number of RT-1 molecules on a rat lymphocyte should be in the range of 60,000 based on the calculated number of 1.9×10^5 β_2 microglobulin molecules present on a rat lymphocyte (Björck et al., 1979). This calculation means that 8000 rat lymphocytes contain approximately 50 pg RT-1, which then is the amount that is detectable in the presented liposome immune assay.

The liposome immune assay can be used as a general method for detection

of antibodies against any kind of membrane structure and for identification and/or quantitation of amphipathic antigens. The method is not limited to membrane proteins as described in this article but can also be used with other membrane components such as lipopolysaccharides. A further advantage with the liposome immune assay is that poorly purified antigens may be used successfully for many purposes. It appears that the insertion of membrane antigen molecules into lipid vesicles is a suitable way of presenting the antigens to the immune system. It may be superior to several other ways of antigen presentation by keeping the tertiary structure of the molecules more akin to that in the original cell membrane, and thereby optimizing the possibilities of demonstrating immune interactions with relevant determinants. The liposome immunoassay appears to be suitable for assaying various membrane preparations for antigens against which specific antibodies are available. Similarly, the method also appears to be quite useful for screening numerous sera for antibodies to specified membrane antigens.

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Lectin-Mediated Binding of Liposome-Inserted Membrane Proteins to Red Blood Cells. A Method to Detect Binding of Antibodies to Purified Rat Histocompatibility Antigen or Binding of Insulin to the Insulin Receptor

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Partially purified membrane proteins such as the rat RT-1 histocompatibility antigen or the insulin receptor of porcine liver were inserted into liposomes. These liposomes then bound efficiently to Con A-coated red blood cells. After attachment of the liposomes to the cells, cell-liposome conjugates could be separated from free liposomes by centrifugation. Binding of FITC-labeled specific antibodies or insulin to membrane proteins inserted into the liposomes could then be analyzed with a cell flow cytofluorometer.

The amount of RT-1 antigen that became associated with the cells depended on the composition and concentration of phospholipids during liposome formation. No association of RT-1 antigen to the cells was obtained in the presence of 50 mM α -methyl mannoside.

The technique allows detection of μg amounts of the histocompatibility antigen associated with the red blood cells. It was possible to detect binding of insulin to cells to which approximately 9 ng (3×10^{-14} mol) insulin receptor/ 10^6 cells had been attached. This amount of membrane protein was close to the detection limit of the method. -

Key words: *liposomes – membrane proteins – histocompatibility antigen – insulin receptor – cytofluorograph*

Introduction

Purification of membrane proteins requires the use of detergents to solubilize the plasma membrane. High concentrations of such detergents interfere with the analysis of the membrane proteins. Reduction of the detergent concentration sometimes makes it possible to analyze binding of peptide hormones to solubilized membrane receptors (Cuatrecasas and Parikh, 1974; Nicholas et al., 1974; Shiu and Friesen, 1974). In these cases the separation of bound and free hormone is achieved by precipitation of the receptor-hormone complex with polyethylene glycol. The detergent can be completely removed from the solution without precipitation of the

membrane proteins if they are inserted into liposomes. After insertion of membrane receptors into liposomes, detection of hormones binding to the liposomes requires time-consuming and laborious methods such as ultra-centrifugation and/or gel filtration. Binding of liposomes to cells, however, facilitates the separation of liposomes from free hormone. Liposomes containing erythrocyte sialoglycoprotein have been bound to cells with different lectins as bridge between cells and liposomes (Juliano and Stamp, 1976). After incubation of the cells with lectin, unbound lectin is removed by centrifugation. The cells are then resuspended and incubated with liposomes. The present investigation demonstrates the possibility of detecting binding of antibodies against the rat histocompatibility antigen (RT-1) or insulin to liposomes containing the RT-1 antigen or the insulin receptor after attachment of the liposomes to red blood cells.

Materials and Methods

Partial purification of histocompatibility antigen (RT-1) from rats of the inbred Wistar-Furth strain

Plasma membranes were prepared from WF rat spleen tissue, solubilized with deoxycholate (DOC) and chromatographed on lentil lectin Sepharose as previously described (Axelsson et al., 1981). Glycoproteins were eluted with 0.1 M α -methyl mannoside (Grade III, Sigma Chemical Co., St. Louis, MO).

Protein determination was carried out according to Lowry et al. (1951) with bovine serum albumin as standard. Eluted glycoproteins were concentrated to 0.54 mg/ml.

Purification of the insulin receptor

The purification procedure was based on the method of Cuatrecasas and Parikh (1974) and Jacobs et al. (1977). Plasma membranes were prepared from 200 g fresh porcine liver and solubilized with 20 ml/l Triton X-100. The receptor was further purified by ion-exchange chromatography on DEAE-cellulose (column size 2.5×14 cm). Peak fractions of insulin-binding activity were pooled and applied to a 2 ml column of insulin-succinyl-diaminodipropylamino-Sepharose. The insulin receptor was eluted with 50 mM sodium acetate buffer containing 1 ml/l Triton X-100 and 4.5 M urea. Fractions of 1 ml were collected and immediately diluted with an equal volume of PBS, containing 2.5 g/l BSA (PBS used in all experiments was Dulbecco's phosphate buffer containing no Ca^{2+} and Mg^{2+} ions).

Insulin-binding activity was measured with the polyethylene glycol assay (Cuatrecasas, 1972). Samples, 50 μ l and 50 μ l PBS, 5 g/l BSA or 50 μ l PBS, containing 5 g/l BSA and 80 μ g/ml native insulin (crystalline from bovine pancreas, Sigma Chemical Co., St. Louis, MO) were incubated with 100 μ l ^{125}I -labeled insulin (0.4 nM, mono- ^{125}I -(Tyr-A14)-insulin, Novo Research Institute, Copenhagen) in 0.1 M Hepes, 0.12 M NaCl, 1.2 mM MgCl_2 , 2.5 mM KCl, 1 mM EDTA and 0.5 mM sodium acetate, pH 7.9 (Hepes-binding buffer) for 60 min at 24°C. After precipitation of the ^{125}I -labeled insulin-insulin receptor complex with the polyethyl-

ene glycol precipitation method, the samples were centrifuged 10 min at $2500 \times g$ and the activities of the pellets measured.

The protein content of the pooled fractions after DEAE-cellulose chromatography was 5.7 mg/ml.

Preparation of lipids

Glycolipids were extracted from *Micrococcus lysodeikticus* as previously described (Lennarz and Talamo, 1966; Sasaki and Sakagami, 1978). Briefly, 1 g of dried cells (Sigma Chemical Co., St. Louis, MO) was suspended in 3 ml 150 mM NaCl and mixed with 60 ml chloroform:methanol (2:1) and incubated with stirring for 150 min at 24°C. Insoluble material was removed by filtration through a Buchner funnel and the extract was washed by the method of Folch et al. (1957). Finally the volume was reduced to approximately 3 ml by evaporation and then adjusted to 6 ml with chloroform.

Two different preparations were made and the content of dry material was 12.5 and 6 mg/ml respectively. After thin layer chromatography on silica gel plates with chloroform:methanol:7 M NH_4OH (12:60:10) as the mobile phase, both preparations showed 2 spots which were detected with iodine vapor (R_f 0.65 and 0.82). No free amino groups or phosphate were detected with 0.1% ninhydrine solution or 'Molybdatophosphorsäure' (Merck, Darmstadt).

FITC labeling of phosphatidylethanolamine

Fluorescein-labeled lipids were prepared as previously described by Huang et al. (1981). Briefly, 8 mg of fluorescein isothiocyanate (FITC isomer I Sigma Chemical Co., St. Louis, MO) were dissolved in 500 μl chloroform containing 5 mg phosphatidylethanolamine (Type III, Sigma Chemical Co., St. Louis, MO). Triethylamine, 30 μl , was added to the solution and the mixture was incubated in the dark for 8 h at room temperature. The fluorescent phosphatidylethanolamine conjugate was twice purified by thin layer chromatography on silica gel plates with the solvent system chloroform:methanol:water (70:25:4). After purification, one spot containing both phosphate and the fluorescence label was detected.

Preparation of mono-FITC-insulin

Mono-FITC-insulin was prepared by a modification of the method described by Bromer et al. (1967). Briefly, 5 mg crystalline bovine insulin were dissolved in 0.5 ml $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer (pH 9.1) and an equimolar amount of FITC was added. The reaction was allowed to continue in the dark for 50 min at 20°C. Unreacted FITC was removed by passing the incubation mixture through a PD-10 column (Pharmacia Fine Chemicals, Uppsala) equilibrated and eluted with 10 mM Tris-HCl, 7 M urea, pH 7.4. The void fraction (1 ml) was applied to a 15 ml DEAE-Sephadex A-25 column (Pharmacia Fine Chemicals), equilibrated with 10 mM Tris-HCl, 7 M urea, pH 7.4. The column was washed with 0.5 ml of the starting buffer before elution of the conjugate with a 60 ml linear NaCl gradient (0–1 M concentration) in running buffer. Fractions containing 1.6 ml were collected. The peak fraction of the mono-FITC-insulin was collected and the urea removed by passage over a column of

Sephadex G-25, which was equilibrated and developed in PBS containing 10 g/l BSA. The mono-FITC insulin had a concentration of 3.8 μ M, calculated using a molecular extinction coefficient at 492 nm of 68,000 (Bromer et al., 1967), and was stored at 4°C after addition of 1 g/l NaN_3 .

Liposome preparations

Liposomes containing RT-1 antigen. Extracted lipids (313 μ g or 150 μ g) from *Micrococcus lysodeikticus* and various amounts (see Fig. legends 3, 4, 5) of phosphatidylcholine (Type V-E, Sigma Chemical Co., St. Louis, MO) or phosphatidylethanolamine (Type III-E, Sigma Chemical Co., St. Louis, MO) were dried under a stream of nitrogen. The lipids were redissolved in 100 μ l 10 mM Tris-HCl, 5 g/l deoxycholate (DOC), pH 8.2, containing 54 μ g RT-1 antigen. The detergent was removed by gel filtration on a Sephadex PD-10 column which was equilibrated and developed with PBS. After eluting the column with 2.6 ml, 1 ml containing liposomes was collected.

Liposomes containing FITC-phosphatidylethanolamine. Lipids extracted from *Micrococcus lysodeikticus* (150 μ g), various amounts (see Fig. legend 6) of phosphatidylcholine or phosphatidylethanolamine and less than 1 weight % phosphatidylethanolamine-FITC, were dried under a stream of nitrogen. The lipids were redissolved in 100 μ l Tris-HCl, 5 g/l DOC, pH 8.2 and applied to a PD-10 column as previously described.

Liposomes containing the insulin receptor. Lipids extracted from *Micrococcus lysodeikticus* (600 μ g/ml after solubilization) and various amounts (see Table II) of phosphatidylethanolamine were dried under a stream of nitrogen. The lipids were redissolved in the eluate from the DEAE-cellulose purification step of insulin receptor (150 μ l of the eluate and 150 μ l PBS containing 2.5 g/l BSA) or in the eluate containing affinity purified insulin receptor (300 or 1200 μ l of the eluate). The detergent, Triton X-100, was removed by adding 250 g/l Biogel SM-2 beads (Bio-Rad, Richmond, CA) and slowly mixing the samples by rotation for 1 h at 20°C. As a blank, liposomes were prepared by dissolving the lipids in PBS containing 2.5 g/l BSA, 1 ml/l Triton X-100 before addition of Biogel SM-2 beads as previously described.

Flow cytometric analysis

FITC-stained cells were analyzed at a concentration of about 10^6 cells/ml, using a Cytofluorograph System 50-H (Ortho Instruments, Westwood, MA) equipped with a 4 W argon-ion laser (Model 95, Lexel, Palo Alto, CA). The laser line at 488 nm (450 mW power output) was used for excitation, and the emission was recorded after passage through a green fluorescence filter combination (515–555 nm). In order to recognize the red blood cell population the cell size was determined by measuring the axial light extinction at the 632.8 nm wavelength using a He-Ne laser (Spec Physics Mountain View, CA), effect 0.8 mW.

The 2 parameters measured were displayed simultaneously as a 2-dimensional dot plot with the green fluorescence signal on the abscissa and cell size (i.e., axial light extinction signals) on the ordinate. With a suitable threshold setting, only the

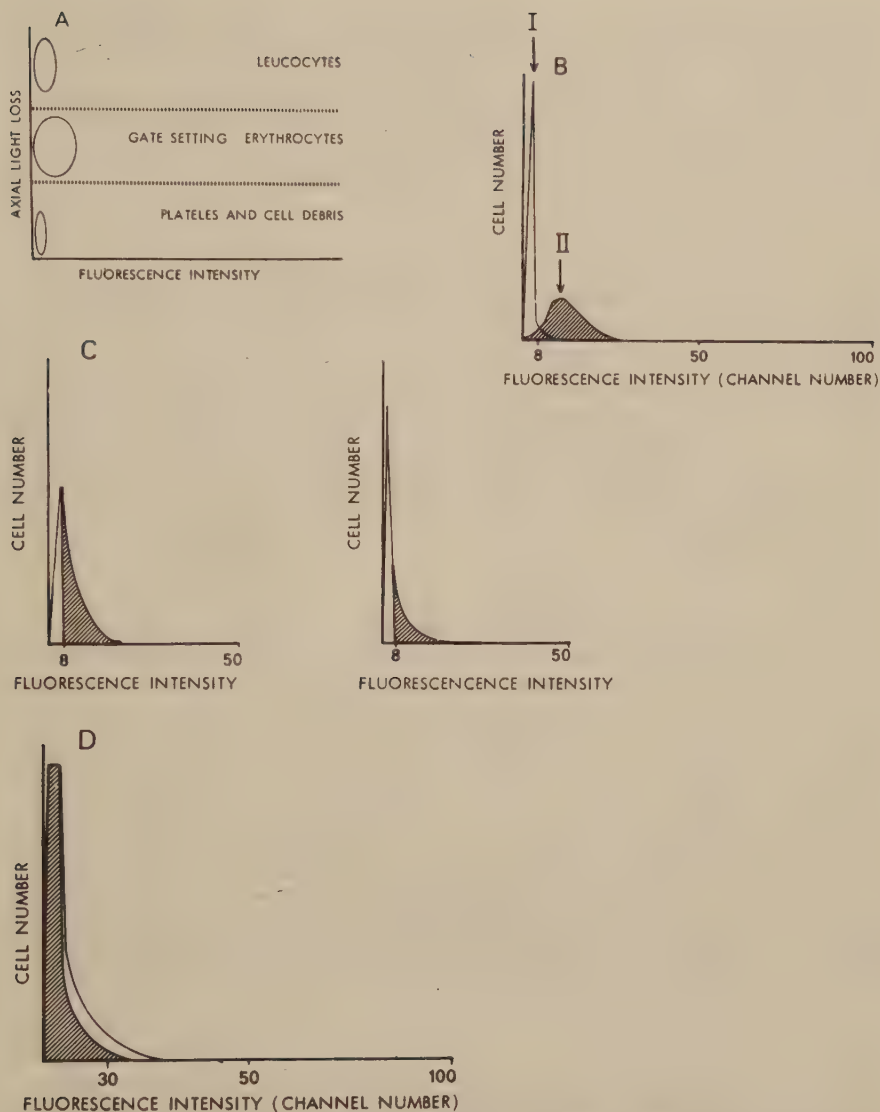


Fig. 1. Scheme of analysis on the Cytofluorograph. A: dot-plot of the cells, showing the gate setting to include only the red blood cells. B: histogram to detect binding of fluorescein-labeled antibodies versus rat histocompatibility antigen. I: peak channel after incubation with normal rat serum. II: peak channel after incubation with antiserum. C: histogram to detect the effect of the lipid composition of glycolipid-liposomes on their association with red blood cells. Left: cells incubated with fluorescein-labeled glycolipid/phosphatidylethanolamine liposomes. Right: cells incubated with fluorescein-labeled glycolipid/phosphatidylcholine liposomes. D: histogram to detect binding of fluorescein-labeled insulin to the insulin receptor. Shaded histogram: cells incubated with liposomes containing no receptor. Unshaded histogram: cells incubated with receptor containing liposomes.

fluorescence signals from the cell population were gated into the multichannel distribution analyzer (512 channels in resolution) and thereafter processed on a computer PDP 11/34 (Digital Equipment Corporation, Maynard, MA). Ten to fifty thousand cells were analyzed in each experiment and the fluorescence intensity of the mean cell population was recorded in the multichannel analyzer as the channel number corresponding to the peak. The analysis on the Cytofluorograph is schematically shown in Fig. 1.

Incubation of cells and Con A to generate a 'receptor' to carbohydrate

Red blood cells (bank blood group O, Rh +) were washed with PBS and suspended in PBS to a concentration between $1.5\text{--}2.5 \times 10^8$ cells/ml. Forty microliters Con A, 26.3 g/l ($2 \times$ crystallized, Miles Biochemicals, U.K.) were added to 750 μ l of the cell suspension and the cells were incubated with gentle mixing for 15 min at 20°C. Unbound Con A was removed from the cells by applying 0.5 ml of the incubate to a 10 ml column of Biogel A-5m (50–100 mesh, Bio-Rad, Richmond, CA). The column was eluted with 2.5 ml PBS before 1 ml was collected. This fraction contained approximately 50% of the applied cells and had a cell concentration of $3\text{--}6 \times 10^7$ cells/ml.

Binding of liposomes to red blood cells

Immediately after gel filtration the cells were added to an equal volume of RT-1 or FITC-phosphatidylethanolamine-containing liposomes and were incubated with gentle mixing for 1 h at 37°C. The cells were washed by adding 100 μ l of the cell-liposome mixture to 5 ml PBS containing 5 g/l BSA (PBS-BSA) at room temperature. The cells were centrifuged for 5 min at $300 \times g$ and the washing procedure was repeated twice with 2 and 1 ml volumes of PBS-BSA respectively. Between each washing the cells were carefully resuspended with a pasteur pipette. Liposomes containing the insulin receptor were mixed in a volume of 250 or 1000 μ l to approximately 1×10^6 cells from which unbound Con A had been previously removed by gel filtration. The cell-liposome mixtures were incubated with gentle stirring for 1 h at 20°C before 5 ml PBS-BSA was added to each mixture and the samples were centrifuged for 5 min at $300 \times g$. The cells were washed once more by carefully resuspending the cells in 2 ml PBS-BSA.

Detection of liposomes bound to the cells

RT-1 containing liposomes. After washing, the cells were resuspended in 50 μ l anti-RT-1 serum or normal rat serum diluted 1 : 20 in PBS containing 10 mM Hepes, 10 g/l BSA, pH 7.4 (PBS-Hepes-BSA) and incubated for 30 min at 4°C. Then 1 ml PBS-BSA was added and the cells were centrifuged. The washing procedure was repeated once more with 1 ml of the same buffer. The pellets were resuspended in 50 μ l FITC-labeled rabbit anti-rat IgG (Dakopatts, Copenhagen) diluted 1 : 10 with PBS-Hepes-BSA and incubated for another 30 min at 4°C. After incubation, the same washing procedure was repeated. Finally the cells were suspended in 1 ml PBS-Hepes-BSA and the mean fluorescence intensity of the cells expressed as the peak channel number determined by a cell flow cytofluorometer.

FITC-phosphatidylethanolamine-containing liposomes. After washing, the cells were resuspended in 1 ml PBS-BSA and the fluorescence of the cells was recorded by a cell flow cytofluorometer.

Insulin receptor liposomes. After washing, the cells were resuspended in 125 μ l PBS-BSA and 50 μ l of the suspension was incubated with gentle mixing for 60 min at 4°C with 50 μ l Hepes binding buffer and 100 μ l mono-FITC-insulin diluted 1:10 with Hepes binding buffer. One milliliter ice-cold PBS-BSA was added and the samples were centrifuged. The washing step was repeated once more with 1 ml PBS-BSA. Finally the cells were resuspended in 1 ml PBS-BSA and the fluorescence from the cells was analyzed on a cell flow cytofluorometer.

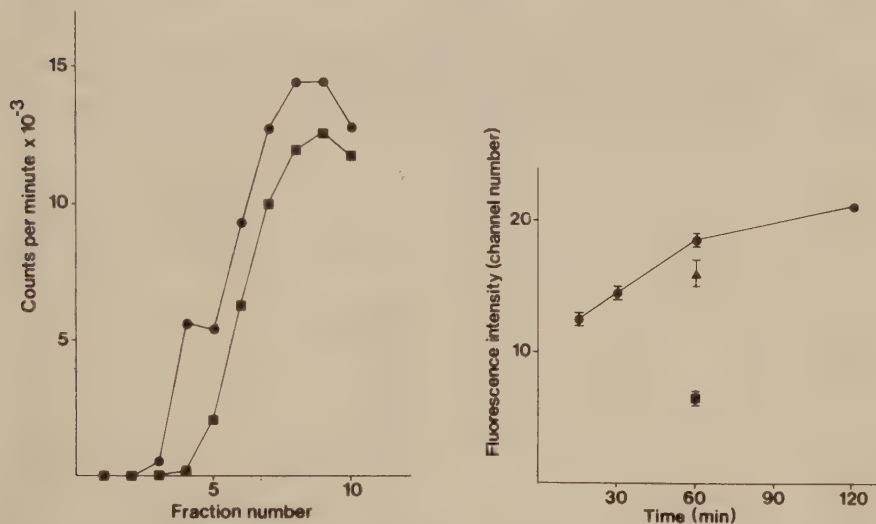


Fig. 2. Elution of 125 I-Con A-incubated red blood cells from a Biogel A-5m column. A suspension of red blood cells, 2.6×10^8 cells/ml (750 μ l) was incubated for 15 min at 20°C with 40 μ l 125 I-Con A (7 μ g, 0.2 μ Ci/ μ g, 8 kBq/ μ g, labeled according to Eriksson et al., 1984). Five hundred microliters of the incubation mixture were applied to a 10 ml column of Biogel A-5m (●). Before use the column was equilibrated with PBS and eluted with the same buffer. Immediately after applying the cells, 1 ml fractions were collected and the radioactivity of 100 μ l of each fraction was measured in a gamma counter. Elution pattern of 125 I-Con A itself (■).

Fig. 3. RT-1 antigen associated with red blood cells after incubation of cells and liposomes for various times. Red blood cells were incubated with Con A and applied to a column of Biogel A-5m. Cells in the void fraction (3.1×10^7 cells/ml) were incubated with gentle mixing at 37°C with an equal volume of liposomes, prepared from 313 μ g extracted lipids, 10 μ g phosphatidylcholine and 54 μ g partially purified RT-1 antigen. At different incubation times, 100 μ l of the cell-liposome mixture were withdrawn, washed with PBS-BSA, incubated with anti-WF serum and stained with FITC-rabbit anti-rat IgG (●). Finally the cells were resuspended in 1 ml PBS-Hepes-BSA and the mean fluorescence intensity of the cells recorded as the peak channel number analyzed on a cell flow cytofluorometer. Cells were incubated with liposomes for 1 h, washed, then incubated with a normal rat serum and stained with FITC-rabbit anti-rat IgG (■). Cells were incubated for 1 h in parallel with liposomes prepared without extracted lipids, washed, incubated with anti-WF serum and stained with FITC-rabbit anti-rat IgG (▲).

Results

A carbohydrate receptor was created on red blood cells by incubation of the cells with Con A. To avoid aggregation of the cells after incubation with this lectin, the unbound Con A was removed by gel filtration of the incubation mixture on a column of Biogel A-5m (Fig. 2). The void fraction from the column contained approximately 50% of the initially applied cells.

After removal of free Con A the cells had the ability to bind liposomes containing the histocompatibility antigen from rats (RT-1) (Fig. 3). The amount of RT-1 antigen bound to the cells increased after prolonged incubation of cells and liposomes. One hour's incubation time was chosen as standard since longer incubation reduced the number of cells recovered.

The liposomes were prepared from phosphatidylcholine, lipids extracted from *Micrococcus lysodeikticus* and partially purified RT-1 antigen. The lipids extracted from *Micrococcus lysodeikticus* contain a dimannosyl diglyceride (Lennarz and

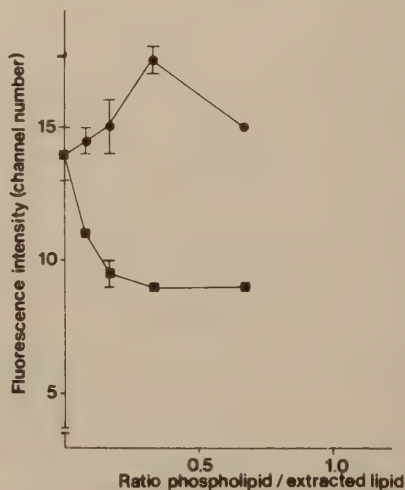
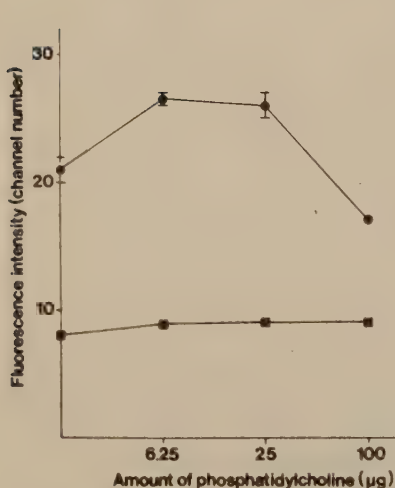


Fig. 4. Binding of RT-1 antigen to cells after preparation of liposomes containing various amounts of phosphatidylcholine. Liposomes were prepared from 313 µg extracted lipids, 54 µg RT-1 antigen and various amounts of phosphatidylcholine. Red blood cells (3.8×10^6 /ml), preincubated with Con A, were incubated with an equal volume (250 µl) of the different liposome preparations. The cells were washed and each mixture was divided into 4 samples, incubated with anti-WF serum (●) or normal rat serum (■) and stained with FITC-rabbit anti-rat IgG. Finally the cells were analyzed on a cell flow cytofluorometer.

Fig. 5. Binding of RT-1 antigen to cells after preparation of liposomes with various amounts of phosphatidylcholine (■) or phosphatidylethanolamine (●). Liposomes were prepared from 150 µg extracted lipids, 54 µg RT-1 antigen and different amounts of phosphatidylcholine or phosphatidylethanolamine. Cells were incubated with liposomes and the amount of RT-1 antigen bound to the cells was analyzed as described in Fig. 3. The amount of phospholipids during liposome formation is expressed as the ratio between the amount of phospholipid and the amount of extracted lipids.

Talamo, 1966) which has been reported to bind to the lectin Con A (Sasaki and Sakagami, 1978). The binding of RT-1 antigen containing liposomes to the cells was only to a minor extent due to the glycolipid content of the liposomes (Fig. 3). The amount of RT-1 antigen bound to the cells was dependent on the concentration of phosphatidylcholine during the formation of liposomes (Fig. 4). Liposomes having a lower concentration of RT-1 antigen were probably formed with phosphatidylcholine concentrations of 1 mg/ml and resulted in a lower amount of RT-1 antigen bound to the cells.

When unbound liposomes were washed away from the cells by centrifugation, the cells aggregated. This indicated that Con A still exposed binding sites for carbohydrates on the cell surfaces after incubation with liposomes and that more liposomes could be bound to the cells. Binding of lectins to glycolipids is increased when the glycolipids are inserted into liposomes composed of charged lipids, compared with liposomes of neutral phospholipids (Hampton et al., 1980; Sundler, 1982). Formation of liposomes with extracted lipids from *Micrococcus lysodeikticus*, RT-1 antigen and the positively charged lipid phosphatidylethanolamine increased the amount of RT-1 antigen bound to the cells (Fig. 5). Formation of liposomes with higher concentrations of phosphatidylethanolamine, giving a higher ratio between phospholipids and extracted lipids, reduced the amount of RT-1 antigen bound to the cells. Omitting the RT-1 antigen in the liposomes gave a lower ratio between the

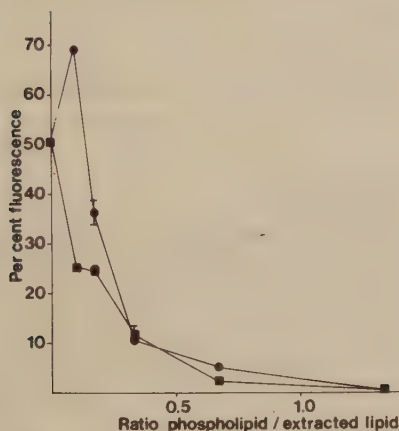


Fig. 6. Binding of liposomes containing no RT-1 antigen to cells. Liposomes were prepared from 150 μ g extracted lipids, trace amounts of FITC-phosphatidylethanolamine and varied amounts of phosphatidylcholine (■) or phosphatidylethanolamine (●). Cells and liposomes were incubated and the fluorescence intensity from the cells was analyzed as described in Methods. The amounts of phospholipids during liposome formation are expressed as the ratio between the amount of phospholipid and the amount of extracted lipids. Cells incubated with lipid vesicles prepared from 150 μ g extracted lipids and trace amounts of FITC-phosphatidylethanolamine had a mean fluorescence intensity corresponding to channel 8. Percent fluorescence calculated as: percentage of the cells found in channel 8 or higher -- percentage of the cells found in channel 8 or higher after incubating cells and liposomes in the presence of 50 mM α -methyl mannoside.

amount of extracted lipids and phosphatidylethanolamine that bound the highest amount of liposomes to the cells (Fig. 6).

The RT-1 antigen was solubilized with the detergent deoxycholate (DOC). This is a detergent that can be removed from the solution during liposome formation by gel filtration or dialysis. Non-ionic detergents usually have too low a critical micelle concentration to be removed by these methods.

The insulin receptor can be solubilized by the non-ionic detergent Triton X-100. Formation of liposomes must therefore be made by adsorption of the detergent to Bioigel SM-2 beads (Holloway, 1973; Volsky et al., 1979). Table I shows the binding of ^{125}I -labeled insulin to receptor purified by affinity or DEAE-cellulose chromatography. Table I also shows the binding to cells which, before incubation with insulin, had been incubated with liposomes containing the receptor at different stages of purification. Cells were exposed to liposomes containing the same amount of DEAE-cellulose-purified receptor as that assayed in the soluble form (Table I). To cells with affinity-purified receptor twice as much was used. Binding of insulin to the cells shows a large variation and an overlap is observed in binding to cells with liposomes containing the receptor and cells with liposomes containing no receptor. If the binding is instead calculated as binding per cell, there is a difference with cells containing DEAE-cellulose-purified receptor liposomes. However, the variation is still large and the overlap still remains with cells containing affinity-purified receptor liposomes.

Another way to measure the binding per cell is to use a cell flow cytofluorometer. This is achieved using a gate setting that measures only the fluorescence of the cells. With an appropriate gate setting a histogram of the cell fluorescence can be obtained. The fluorescence intensity is expressed as 512 channels in the histogram. A

TABLE I

BINDING OF ^{125}I -LABELED INSULIN TO THE INSULIN RECEPTOR

Liposomes were prepared with extracted lipids and 400 μg phosphatidylethanolamine/ml. The liposomes were bound to the cells by incubating 3×10^6 cells with 500 μl liposomes. Fifty microliter samples were incubated for 60 min at 24°C with 50 μl PBS-BSA and 100 μl ^{125}I -labeled insulin (400 pM in Hepes-binding buffer). Binding of solubilized receptor was determined by the polyethylene glycol assay. Red blood cells were diluted with 1 ml ice-cold PBS containing 5 g/l BSA, centrifuged for 5 min at $300 \times g$ and the activity in the pellets measured. Tubes containing 20 μg /ml of non-radioactive insulin were incubated in parallel to determine the extent of non-specific binding. All assays were performed in duplicates and the data corrected for non-specific binding.

Sample	Measured binding (cpm)	Normalized binding/ 10^6 cells
Soluble, DEAE-cellulose-purified receptor	1939 ± 14	
Soluble, affinity-purified receptor	265 ± 164	
Liposomes without receptor, bound to red blood cells	516 ± 314	1229 ± 748
Liposomes containing DEAE-cellulose-purified receptor, bound to red blood cells	1051 ± 404	5003 ± 1922
Liposomes containing affinity-purified receptor, bound to red blood cells	1030 ± 404	3028 ± 1187

TABLE II

BINDING OF MONO-FITC-INSULIN TO LIPOSOMES BOUND TO RED BLOOD CELLS ^a

Experiment	Concentration phosphatidylethanol- amine during liposome preparation	Amount of fluorescence (%) ^b	
		Liposomes containing the receptor	Liposomes without the receptor
<i>Liposomes containing DEAE-cellulose purified receptor ^c</i>			
I	500	3.2	0.2
		2.0	0.3
II	500	1.7	0.5
		1.1	0.3
III	0	1.2	0.1
		1.4	0.1
<i>Liposomes containing affinity purified receptor ^d</i>			
I	250	0.28	0.06
		0.26	0.18
II	0	0.28	0.16
		0.16	0.18

^a Liposomes were prepared, bound to red blood cells and incubated with mono-FITC-insulin as described in Methods.

^b Percentage of the cells having a higher fluorescence intensity than channel 30 on the cell flow cytofluorometer.

^c Binding of liposomes to the cells was performed by incubating 1×10^6 cells with 250 μ l liposomes.

^d Binding of liposomes to the cells was performed by incubating 1×10^6 cells with 1000 μ l liposomes.

clear difference between cells with liposomes containing the DEAE-cellulose purified receptor and cells with blank liposomes was obtained if the percentage of cells having a higher fluorescence intensity than channel 30 was calculated (Table II). The volume of soluble DEAE-cellulose-purified receptor corresponded to 50 μ l per sample. Increasing the volume of affinity-purified receptor to 500 μ l soluble receptor per sample indicated a higher binding to cells with liposomes containing the receptor.

Experiments with the RT-1 antigen had shown an increased binding of antigen to the cells after insertion into liposomes of phosphatidylethanolamine. No increased binding of the insulin receptor to the cells due to phosphatidylethanolamine was observed.

Discussion

Several membrane proteins have been purified and inserted into artificial lipid bilayers. In cases where the biological functions of the membrane proteins are known, these functions are often restored when the membrane proteins are inserted into liposomes.

In order to investigate the function of inserted membrane proteins, the liposomes have to be separated from an incubation mixture. One way of achieving separation is

to attach the liposomes to 'carrier' cells. Lectins with more than 1 binding site for carbohydrates can be used to attach glycoprotein or glycolipid bearing liposomes to cells. The lectin Con A has 4 binding sites for carbohydrates and binds to carbohydrate structures on red blood cells. Aggregation of the cells can be avoided by incubating the cells with an excess of lectin. Due to multipoint attachment on the cell surface, the association constant increases (Emerson and Juliano, 1982) and the unbound fraction of the lectin can be removed without setting up the same equilibrium between free and bound lectin as before. The free lectin must be removed since otherwise no binding of liposomes to the cells is obtained.

Liposomes containing glycolipids or glycoproteins can be bound to the Con A cells. If RT-1 antigen was inserted into mixed liposomes composed of phosphatidylcholine and glycolipids, the liposomes containing RT-1 antigen were mainly bound to the cells, due to interaction between lectin and glycoprotein (Fig. 3).

Lectins have been reported only to bind carbohydrates that extend beyond the phospholipid head groups on the vesicle surface (Slama and Rando, 1980). Since the RT-1 antigen is a glycosylated polypeptide of approximate 45,000 molecular weight the carbohydrate residues of the antigen would be expected to extend beyond the phospholipid head group. Increasing amounts of RT-1 antigen were bound to the cells when the antigen was inserted into liposomes containing phosphatidylethanolamine (Fig. 4). Glycolipids have been reported to bind to the lectin Con A because of a less hydrated head group of phosphatidylethanolamine (Sundler, 1982). The increased binding of RT-1 antigen might be due to better exposure of the carbohydrate residues of the polypeptide or enhanced exposure of the glycolipid resulting in an increased frequency of multipoint attachment of the liposomes to the cells. A multipoint attachment would increase the amount of RT-1 antigen binding since, after incubation with liposomes, the cells were subjected to 3 washes to remove loosely bound liposomes.

The ratio between phosphatidylethanolamine and extracted lipids that increased the binding of liposomes to cells was reduced by omitting the RT-1 antigen in the liposomes (Figs. 4 and 5). This indicates that the exposure of RT-1 antigen was influenced by the surrounding lipids in the liposomes. Binding of liposomes containing phosphatidylethanolamine depended on interactions with Con A and not on the charge of the liposomes since only minor amounts of liposomes were bound to the cells in the presence of 50 mM α -methyl mannoside (not shown).

The insulin receptor is a glycoprotein that binds to Con A (Cuatrecasas and Parikh, 1974) and the purification of the receptor is well documented. The receptor was used as a model to clarify the possibilities of binding liposomes with low receptor content to cells. The receptor content was approximately 90 ng/ml after DEAE-cellulose purification and 1 ng/ml after affinity purification. This was calculated from the binding of solubilized receptor to 125 I-labeled insulin, using 300,00 as the molecular weight of the receptor (Jacobs et al., 1977).

The binding of the insulin receptor to cells was not affected by incorporation of phosphatidylethanolamine into the liposomes. The liposomes were not prepared by the same method as the RT-1 antigen containing liposomes. During the formation of insulin receptor liposomes, BSA was present to avoid adsorption to the SM-2 beads.

Addition of BSA to uncharged liposomes containing glycophorin increases the binding of lectin (Keits and Grant, 1982). Whether binding of liposomes to the cells is affected by adsorption of BSA to the liposomes is now being investigated.

In conclusion, binding of liposomes to cells may be used to separate a membrane protein-ligand complex from free ligand. Different membrane proteins can be bound to cells by lectins having different binding specificities (Lis and Sharon, 1981). Binding of purified membrane components to cells is also an important step when inserting a new membrane protein into the plasma membrane of recipient cells (Eriksson et al., 1984).

Instead of using red blood cells as liposome carriers, Sephadex or other spheres of carbohydrate matrices may be used. A more stable carrier of the liposomes and better detection of the fluorescence signal from the fluorescein molecule would then be expected since hemoglobin absorbs light in the emission region of fluorescein.

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VI

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A sensitive method to introduce membrane-bound proteins into recipient cells based on affinity enrichment of lipid vesicles to the recipient cell prior to fusion

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A sensitive analytical procedure for studying membrane-bound structures has been developed. Membrane glycoproteins inserted into liposomes were transferred to recipient cells by use of a lectin, concanavalin A, bound to the cells as a bridge to generate proximity between the recipient cell and the glycoprotein-containing liposome, prior to exposure to the fusing agent, poly(ethylene glycol). Partially purified histocompatibility antigen from rats was introduced into the membrane of human lymphocytes. After treating the cells with poly(ethylene glycol) under fusion conditions, some of the antigen present in the preparation could not be eluted with α -methyl mannoside and EDTA, indicating that incorporation in the cell membrane had taken place. This antigen remained exposed on the lymphocyte surface for approximately 1 h as demonstrated by sensitivity of the lymphocytes to the lytic effect of an antiserum to the histocompatibility antigen in the presence of complement. Some of the lectin molecules seemed to be internalized in the cells but no induction of cell mitosis was observed. The described method gives an opportunity to work with small amounts of membrane proteins inserted into liposomes, introducing them into recipient cells for analysis of their biological activities.

Introduction

In laboratory work, membrane proteins often present particular problems due to their lability when removed from the environment of phospholipids in the membrane and, also, because of the hydrophobic character of parts of the molecules. These properties have made it difficult to study and/or quantitate membrane proteins in a reliable way. In this context, however, liposome technology seems to offer very promising possibilities.

Membrane preparations can be introduced into foreign cells by fusion of plasma membranes or liposomes with recipient cells without severely af-

fecting the viability of the cells. Poly(ethylene glycol), added to a mixture of cells and plasma membrane vesicles or liposomes, has been reported to cause fusion of cells with the vesicles [1–5]. Fusion of cells and liposomes containing reconstituted membrane proteins has also been reported with the use of Sendai virus glycoproteins [6–8]. These initial and very important papers indicated a possibility to transfer membrane proteins to receptor cells, but they never dealt with transfer of small amounts of membrane proteins, and those experiments were not designed to push the sensitivity of the system. When dealing with enriched preparations of a certain membrane structure, one has a markedly reduced amount available in the fusion

step and the efficiency in the subsequent fusion is consequently put under pressure.

The more purified a membrane preparation to insert is, the higher demands it puts on the fusion process. This paper addresses some of the key questions in this connection.

The present investigation was performed to develop a sensitive procedure by which small amounts of membrane proteins may be transferred to recipient cells. The general principle is to specifically enrich the membrane protein containing lipid vesicles in close proximity to the acceptor cells by use of biospecific interaction with a lectin artificially prelocalized on the cell surface.

As a model system, the introduction of new antigens into human lymphocytes was studied to confirm that they kept their properties after the transfer. As antigen we chose the rat histocompatibility antigen (RT-1) which was partially purified prior to incorporation into lipid vesicles.

Materials

Phosphatidylcholine type V-E, phosphatidylethanolamine type III, dried cells of *Micrococcus lysodeikticus*, α -methyl mannoside grade II, lactoperoxidase 80–100 units/mg, fluorescein isothiocyanate (FITC) isomer I and 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-chloride) were obtained from Sigma Chemicals Co., U.S.A.

Ficoll-Paque, lentil lectin Sepharose and Sephadex G-25 (PD-10 columns) were purchased from Pharmacia Fine Chemicals AB, Sweden and Bio-Gel P-4 (medium) and Bio-Gel A-5m (50–100 mesh) from Bio-Rad, Richmond, U.S.A. Concanavalin A 2 $\times$ crystallized, and bovine serum albumin Cohn fraction V were supplied from Miles Biochemicals, Slough, U.K. and poly(ethylene glycol) 1540, pharmaceutical grade, was from Polysciences Inc., Warrington, U.S.A.

Silica gel plates (Kieselgel 60 ohne Fluoreszenzindikator), 0.1% ninhydrin and molybdatophosphoric acid spray reagents came from Merck, Darmstadt, F.R.G. and the dyes acridine orange 'GURR' and ethidium bromide from BDH, Poole, U.K.

Sodium iodine (Na^{125}I) for protein iodination 3.7 GBq/ml (100 mCi/ml) was purchased from the Radiochemical Centre, Amersham, U.K. and

[methyl- ^3H]thymidine (2 Ci/mmol) was from New England Nuclear, Boston, U.S.A.

FITC-labeled rabbit anti-rat IgG was obtained from DAKOPATTS a/s, Copenhagen, Denmark and rabbit anti-concanavalin A from E·Y Laboratories Inc., San Mateo, U.S.A. Antiserum against the rat strain Wistar-Furth (WF) was produced by immunization of adult animals of the Brown Norway (BN) strain, with three biweekly injections intraperitoneally of $2 \cdot 10^7$ allogenic spleen and lymphnode cells.

Fresh serum of guinea pigs was frozen and stored at -80°C , thawed and used as the source of complement. RPMI 1640 was supplied from Flow Laboratories, Solna, Sweden.

Phosphate-buffered saline used in the experiments was Dulbecco's phosphate buffer containing no Ca^{2+} or Mg^{2+} .

Methods

Lymphocyte preparation

Human peripheral blood lymphocytes from healthy donors of blood type O, Rh $^+$, were purified by Ficoll-Hypaque centrifugation [9] and depleted of monocytes on a collagen column [10]. The cells were washed with phosphate-buffered saline and suspended in the buffer to cell concentrations of approximately $(1-2) \cdot 10^8$ cells/ml.

Partial purification of histocompatibility antigen (RT-1) from rat of the inbred strain Wistar-Furth (WF)

Plasma membranes of spleen tissue of WF rats were prepared, solubilized with deoxycholate and affinity-separated on lentil lectin Sepharose as previously described [11,12]. Protein determination was carried out according to Lowry et al. [13], using bovine serum albumin as a standard. After solubilization of the plasma membranes, the protein concentration was 7.2 mg/ml. Eluted glycoproteins were concentrated to 0.54 mg/ml.

Preparation of lipids

Glycolipids were extracted from *Micrococcus lysodeikticus* as previously described [14–16].

Fluorescence labeling of phosphatidylethanolamine was prepared by dissolving 4 mg of NBD-chloride in 500 μl chloroform containing 5

mg phosphatidylethanolamine and 15 μ l of triethylamine. The mixture was incubated in the darkness for 8 h at room temperature. The fluorescent phosphatidylethanolamine conjugate was purified twice by thin-layer chromatography on silica plates using the solvent system chloroform/methanol/water (70:25:4, v/v). After purification, one fluorescent and phosphate-containing spot was observed. The conjugate was finally dissolved in 2.5 ml chloroform/methanol/water (70:25:4, v/v).

¹²⁵I-labeling of concanavalin A

The lectin was labeled with Na¹²⁵I according to the lactoperoxidase method [17]. 25 μ l concanavalin A, 2.6 mg/ml in phosphate-buffered saline were added to 10 μ l Na¹²⁵I (37 MBq). 2 μ l lactoperoxidase (2 mg/ml) and 10 μ l H₂O₂ (0.003%, v/v) were added under magnetic stirring. After 15 s the iodination was stopped by adding 0.4 ml phosphate-buffered saline. The unbound ¹²⁵I was separated by passing the preparation through a Bio-Gel P-4 column (10 $\times$ 1 cm). The fraction eluted in the void volume was collected and stored at 4°C. The lectin had a specific activity of 10 μ Ci/ μ g (400 kBq/ μ g).

Lipid vesicle preparation

Lipid vesicles containing the RT-1 antigen were prepared as previously described [16] by removal of the detergent by gel filtration on Sephadex G-25 (PD-10 columns).

Unless otherwise stated, 1 ml of vesicles were prepared from 313 μ g lipids extracted from *Micrococcus lysodeikticus* and 54 μ g glycoproteins eluted from lentil lectin Sepharose.

Flow cytometric analysis

FITC-stained cells were analyzed at a concentration of about 10⁶ cells/ml, on a Cytofluorograph System 50-H (Ortho Instruments, Westwood, MA) as previously described [16]. With an appropriate setting, only the fluorescence signals from the lymphocyte population were gated into the multichannel distribution analyzer with a setting of 512 channels in resolution. 10 000–50 000 lymphocytes were analyzed in each experiment and the fluorescence intensity from the mean cell population was determined as the channel number on the multichannel analyzer corresponding to the peak.

Experimental procedure to transfer the RT-1 antigen to lymphocytes

Incubation of cells and concanavalin A to generate a 'receptor' for carbohydrates. Suspended lymphocytes, containing (1–2) $\cdot$ 10⁷ cells, in a volume of 100 μ l were mixed with 40 μ l of concanavalin A solution (16 μ g/ml) and incubated for 15 min, at 20°C.

Binding of lipid vesicles containing RT-1 antigen to the cells. After dilution with 910 μ l phosphate-buffered saline, 1 ml of the cell suspension was added to 0.5 ml lipid vesicles.

Addition of poly(ethylene glycol) to obtain fusion conditions. The cell-vesicle mixture was incubated for 1 h at 37°C on a rocking-table and 1 ml poly(ethylene glycol) 1540 (45% w/w in phosphate-buffered saline) was added to 100 μ l of the cell-vesicle mixture with gentle and continuous mixing over a 1 min time period.

Elution of concanavalin A bound to the cells. 30 s later, 5 ml of phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin, 0.1 M α -methyl mannoside and 10 mM EDTA was slowly added over a period of 2 min. The cells were then sedimented by centrifugation for 5 min at 200 $\times$ g and resuspended in 2 ml phosphate-buffered saline, bovine serum albumin, α -methyl mannoside and EDTA. This washing of the cells was repeated once more with 1 ml phosphate-buffered saline, bovine serum albumin, α -methyl mannoside and EDTA. All washing steps were performed with buffer solutions prewarmed to 37°C.

After washing of the cells, the pellets were incubated 30 min at 4°C with anti-WF serum or normal BN serum diluted 1:20 in phosphate-buffered saline containing 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 1% (w/v) bovine serum albumin, pH 7.4 (buffer 1). The cells were washed twice in phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin (buffer 2), stained with FITC-labeled rabbit anti-rat IgG, washed, suspended in 1 ml buffer 1 and the fluorescence from the cells was assayed on a cell flow cytofluorometer.

The proportion of viable cells was recorded after staining with a mixture of the nucleic acid-specific dyes, acridine orange and ethidium bromide [18].

Detection of concanavalin A not eluted from the cells

Lymphocytes ($100\ \mu\text{l}$ of $15 \cdot 10^7$ cells/ml) were incubated with $40\ \mu\text{l}$ ^{125}I -labeled concanavalin A ($16\ \mu\text{g}/\text{ml}$, $10\ \mu\text{Ci}/\mu\text{g}$) for 15 min at 20°C . The cells were diluted in $910\ \mu\text{l}$ phosphate-buffered saline and added to $500\ \mu\text{l}$ of phosphate-buffered saline instead of lipid vesicles. $1\ \text{ml}$ of phosphate-buffered saline or 30% and 45% poly(ethylene glycol) solution, respectively, was added to $100\ \mu\text{l}$ of the cell suspension and the cells were washed three times with either buffer 2 or phosphate-buffered saline, bovine serum albumin, α -methyl mannoside and EDTA. The rest of the cell suspension was incubated for 1 h at 37°C before treatment with poly(ethylene glycol) and washing in the same manner as previously described. The radioactivity of washed cells was measured and normalized as cpm/ 10^6 cells and the percentage binding sites on the cells still occupied by the lectin was calculated.

Lymphocytes ($100\ \mu\text{l}$ of $17 \cdot 10^7$ cells/ml) were incubated with $40\ \mu\text{l}$ concanavalin A ($16\ \mu\text{g}/\text{ml}$). The cells were treated as after incubation with ^{125}I -labeled concanavalin A. After washing, the pellets were incubated for 30 min at 4°C with rabbit anti-concanavalin A antibodies diluted 1:20 in buffer 1. The cells were washed twice with buffer 2 and stained with FITC-labeled goat anti-rabbit IgG, prepared according to Huang et al. [19]. After washing, the cells were suspended in $1\ \text{ml}$ buffer 1 and analyzed on a cell flow cytofluorometer.

Assay of mitogenic activity

The RT-1 antigen was transferred to lymphocytes from vesicles prepared from $313\ \mu\text{g}$ extracted lipids and $54\ \mu\text{g}$ eluted glycoproteins. One sample of the cell-vesicle mixture was untreated and another sample was treated with 45% poly(ethylene glycol). The samples were washed with 5, 2 and $1\ \text{ml}$ phosphate-buffered saline containing bovine serum albumin, α -methyl mannoside and EDTA and once with $5\ \text{ml}$ RPMI 1640 supplemented with 10% fetal calf serum to remove remaining α -methyl mannoside and, finally, were resuspended in the same medium. $200\ \mu\text{l}$ lymphocytes ($1 \cdot 10^6/\text{ml}$) were added per well of a microtiter plate and were incubated for 72 h at 37°C in an atmosphere containing 8% CO_2 , with or without

concanavalin A ($8\ \mu\text{g}/\text{ml}$). The cells were pulsed for 8 h with [*methyl*- ^3H]thymidine ($1\ \mu\text{Ci}/\text{well}$) and then transferred to filters and were washed and dried before being counted in a liquid scintillation counter. As a comparison, lymphocytes were washed with buffer 2 or phosphate-buffered saline with bovine serum albumin, α -methyl mannoside and EDTA prior to washing with RPMI 1640 supplemented with 10% fetal calf serum and subsequent incubation in microtiter wells under the conditions previously described.

Complement lysis of the cells

After transfer of RT-1 antigen into lymphocytes, ten preparations of cells with associated RT-1 antigen were pooled and suspended in $1.1\ \text{ml}$ RPMI supplemented with 10% fetal calf serum. To $50\ \mu\text{l}$ of the cells were added $50\ \mu\text{l}$ of each dilution of an anti WF-serum serially diluted in RPMI supplemented with 10% fetal calf serum. The cells were incubated for 30 min at 4°C and washed with $1\ \text{ml}$ buffer 2. After careful resuspension of the cells, $50\ \mu\text{l}$ active or heat inactivated ($30\ \text{min}$, 57°C) guinea pig complement diluted 1:1 with buffer 1 were added, and the cells were incubated for 45 min at 37°C . The cells were sedimented and resuspended in $1\ \text{ml}$ phosphate-buffered saline containing acridine orange and ethidium bromide ($0.5\ \mu\text{g}$ of each dye), and the number of viable cells was analyzed on a cell flow cytofluorometer [18].

Results

Determination of concanavalin A and poly(ethylene glycol) concentrations suitable for transfer of lipid vesicles to the surface membranes of lymphocytes

A 'cell-bound receptor' for carbohydrates was generated by incubating cells and concanavalin A. High concentrations of concanavalin A aggregated the cells and increased the ability of cell-cell fusion after addition of poly(ethylene glycol). The degree of cell aggregation after incubation with various concentrations of concanavalin A was determined as a decrease in the number of cells being able to pass through a column of Bio-Gel A-5m (Fig. 1). In the subsequent assay, $4.6\ \mu\text{g}$ concanavalin A/ml was used. The largest amount of fluorescent vesicles associated with cells without an increased cell death

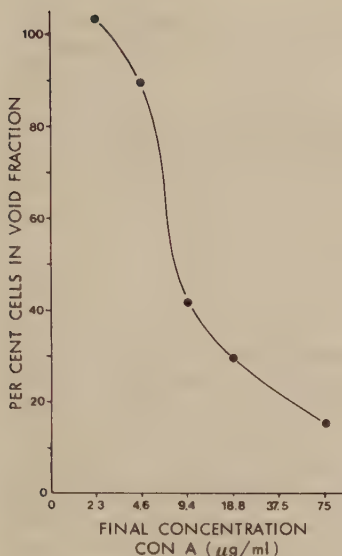


Fig. 1. Incubation of lymphocytes and concanavalin A, con A. 500 μ l cell suspension ($18 \cdot 10^7$ cells/ml) were incubated with 200 μ l concanavalin A in concentrations varying from 8.2 to 263 μ g/ml. After 15 min at 20°C, 500 μ l of the incubation mixture were applied to a 10 ml column of Bio-Gel A-5m. Prior to use, the column was equilibrated with phosphate-buffered saline and the cells were eluted with the same buffer. 1-ml fractions were collected. The largest cell number was obtained in fraction four and the number of cells in this fraction was counted and expressed as percentage of cells recovered without incubation with concanavalin A. A new column was used for each concanavalin A concentration.

was obtained after treatment with 45% (w/w) poly(ethylene glycol) (Fig. 2). At higher poly(ethylene glycol) concentrations there was a dramatically increased lysis of the cells. Fluorescence of higher intensity was obtained when cells had been preincubated with concanavalin A. Some of the fluorescence was only loosely bound to the cells and could be removed by treatment with a combination of α -methyl mannoside and EDTA (Fig. 2).

Transfer of rat histocompatibility antigen (RT-1) to human lymphocytes

Approximately the same amount of cells was

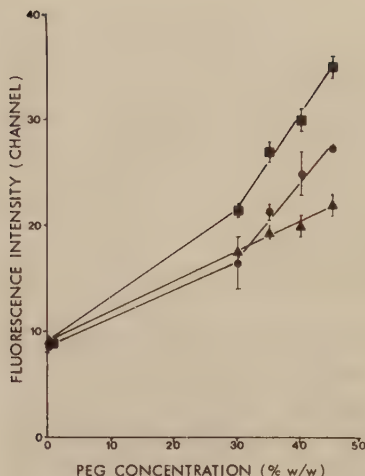


Fig. 2. Fluorescence associated with the cells after incubation with lipid vesicles containing fluorescent lipids. Lymphocytes, 100 μ l of a suspension containing $14 \cdot 10^7$ cells/ml were incubated for 15 min at 20°C with 40 μ l concanavalin A (16 μ g/ml) or phosphate-buffered saline. The cells were diluted with 910 μ l phosphate-buffered saline, and 1 ml of the cell suspension was added to 0.5 ml vesicles prepared from 625 μ g lipids from *Micrococcus lysodeikticus*, 50 μ g phosphatidylethanolamine-NBD conjugate and solubilized plasma membranes corresponding to 180 μ g protein. After 60 min at 37°C, 1 ml of phosphate-buffered saline or 1 ml of 30, 35, 40 or 45% (w/w) poly(ethylene glycol) (PEG) was added to 100 μ l of the cell-vesicle incubates. Cells incubated with concanavalin A prior to addition of lipid vesicles were either washed with phosphate-buffered saline and 0.5% (w/v) bovine serum albumin (■) or the same buffer containing 0.1 M α -methyl mannoside and 10 mM EDTA (●). Cells not incubated with concanavalin A were washed with phosphate-buffered saline containing 0.5% (w/v) bovine serum albumin (▲).

recovered from the transfer procedure with or without addition of poly(ethylene glycol) (85% of the cells). However, after poly(ethylene glycol) treatment 10–15% of the recovered cells were dead. Poly(ethylene glycol) treatment in combination with lipid vesicles increased the percentage of dead cells to 20–25%.

After mixing cells and lipid vesicles with no poly(ethylene glycol) added, none or only a very small amount of RT-1 antigen was detected on the cells (Table I), as reflected by no increased fluores-

TABLE I

For the cell-associated fluorescence, results are given as the channel number corresponding to the mean fluorescence intensity in the multichannel analysis on a cell flow cytometer. PBS, phosphate-buffered saline; BSA, bovine serum albumin; Con A, concanavalin A; PEG, poly(ethylene glycol).

Cells exposed to vesicles containing the antigen		Cell-associated fluorescence	
Further treatment	Washing	Anti-WF serum	BN-normal serum
~	PBS (0.5% BSA)	17	12
Pretreatment with Con A	α -methyl-mannoside + EDTA	21	13
Subsequent PEG incubation	PBS (0.5% BSA)	22	14
Pretreatment with Con A and subsequent PEG incubation	α -methylmannoside + EDTA	27	15

cence above the normal background difference of four channels between antiserum and normal BN rat serum. Association of antigen with the cells was detected when the mixture of cells and lipid vesicles was treated with poly(ethylene glycol) under fusion conditions. Incubation of the cells with concanavalin A prior to the treatment with lipid vesicles and poly(ethylene glycol) resulted in a larger amount of antigen associated with the cells. Omitting the poly(ethylene glycol) treatment reduced the amount of detectable antigen.

Amount of concanavalin A remaining on the lymphocytes after washing

125 I-labeled concanavalin A or an antiserum against the lectin was used to investigate whether

or not concanavalin A was still present on the cells after washing with α -methyl mannoside and EDTA (Table II). Both methods showed that the amount of lectin that could be removed from the cells decreased after incubation at 37°C. This effect was most pronounced when antibodies were used to detect remaining lectins. Treatment of the cells with higher concentrations of poly(ethylene glycol) increased the amount of lectin which became associated with the cells.

A Steck-Wallach plot [20] was used to determine the maximal amount of labeled lectin that can be bound to the cells. Analysis of three different cell preparations showed that the maximal amount of concanavalin A that could be bound to the cells was 485047 ± 22679 cpm per 10^6 cells.

TABLE II

CONCAVALIN A REMAINING ON HUMAN LYMPHOCYTES AFTER FUSION TREATMENT. DETECTION WITH ANTIBODIES AGAINST CONCAVALIN A OR WITH 125 I-LABELED CONCAVALIN A

Cells were treated as described in Methods under 'Detection of concanavalin A not eluted from cells'. The results for the cell-associated fluorescence (A) are given as the channel number, in the multichannel analysis on a cell flow cytometer, corresponding to the mean fluorescence intensity. Cells incubated with antibodies but not with concanavalin A were registered in channel 5. The percentages of binding sites occupied (B) were calculated from the binding of 125 I-labeled concanavalin A. PEG, poly(ethylene glycol).

PEG concn. (% w/w)	Incubation for 15 min at 20°C, washing conditions		Subsequent 60 min at 37°C, washing conditions	
	Eluting	Non-eluting	Eluting	Non-eluting
A. Cell-associated fluorescence				
0	6	60	9	34
45	9	88	13	39
B. Percentage of binding sites occupied				
0	1.7	7.3	4.6	6.3
45	1.9	8.8	4.7	7.1

This value was used to calculate the percentage binding sites on cells still occupied by lectin after washing under eluting or non-eluting conditions (Table II).

Since concanavalin A bound to the cells could not be completely eluted, a study of whether or not this polyclonal T-cell activator [21] might induce cell mitosis under the conditions of these experiments was undertaken. There was no augmented incorporation of [^3H]thymidine into the cells caused by remaining lectin. Less than 1000 dpm of [^3H]thymidine was incorporated into the cells after incubation for 72 h followed by pulsing for 8 h with 1 μCi [^3H]thymidine. The optimal concentration of this lectin preparation for stimulating the incorporation of [^3H]thymidine into cells was found to be 8 $\mu\text{g}/\text{ml}$. This concentration of concanavalin A induced incorporation of $65\,309 \pm 3\,806$ dpm into untreated cells. Cells washed with α -methyl mannoside and EDTA, and cells incubated with concanavalin A and lipid vesicles also washed with α -methyl mannoside and EDTA showed almost the same amount of incorporation of [^3H]thymidine as untreated cells: $70\,537 \pm 3\,510$ and $79\,993 \pm 10\,342$ dpm, respectively. However, when the cells were treated with lipid vesicles under fusion conditions, $33\,607 \pm 3\,220$ dpm were incorporated into the cells after restimulation with concanavalin A.

Transfer of rat histocompatibility antigen (RT-1) results in sensitivity to complement-dependent cytotoxicity by anti-RT-1 antibodies

After transfer of the RT-1 antigen to human lymphocytes these cells became susceptible to lysis by anti-WF serum in the presence of complement. Fig. 3 shows the percentage of dead cells after incubation with various dilutions of the anti-WF serum. Lysis of the cells due to antiserum and complement rapidly decreased at antiserum dilutions higher than 1:10, but was still detectable at a dilution of 1:80.

These results were obtained with cells tested immediately after they had been treated with poly(ethylene glycol) and washed with α -methyl mannoside and EDTA. In contrast, no significant cell lysis was obtained when the cells were stored in RPMI 1640 supplemented with 10% fetal calf serum at 4°C for 60 min prior to exposure to

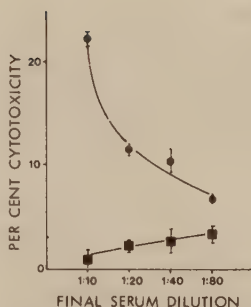


Fig. 3. Complement lysis of cells after transfer of RT-1 antigen. After poly(ethylene glycol) treatment and washing, the cells were incubated with varied dilutions of anti-WF serum and active complement (●) or heat-inactivated complement (■). Percent cytotoxicity of the RT-1 serum is calculated as: $100 \times (C - CS)/C$ (C = % viable cells with complement alone, CS = % viable cells with serum and complement). Percent viable cells with complement alone was 62%.

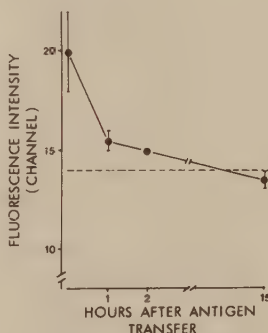


Fig. 4. Elimination of the RT-1 antigen after transfer to the cells. The RT-1 antigen was attached to lymphocytes as described in methods. Ten samples of cells with transferred RT-1 antigen were pooled and suspended in 1 ml RPMI 1640 supplemented with 10% fetal calf serum ($10.2 \cdot 10^6$ cells/ml) and stored at 4°C. At varying times after suspending the cells, 50 μl of the cells were incubated anti WF serum. The cells were washed and stained with FITC-rabbit anti-rat IgG and analysed on a cell flow cytometer. The channel on the multichannel analyzer corresponding to the fluorescence intensity of the mean cell population is presented as a measure of the amount of remaining antigen. Immediately after suspending the cells in RPMI 1640 supplemented with 10% fetal calf serum, cells incubated with phosphate-buffered saline instead of liposomes had a fluorescence intensity that corresponded to channel 14 (— —).

antiserum and complement. A more sensitive way to investigate the disappearance of the antigen from the cell surface was to incubate the cells with an antiserum and a FITC-labeled second antibody. This method revealed that the antigen disappeared from the cell surface within few hours (Fig. 4).

Discussion

Poly(ethylene glycol) concentrations of 40% or higher are required for the fusion of cells [22]. These concentrations have also been used to fuse cells and membrane vesicles [1-3]. Increasing amounts of fluorescent liposomes were associated with the cells after adding increasing concentrations of poly(ethylene glycol) to the cell-vesicle mixtures (Fig. 2). The yield of the fusion process continued to increase when 50% poly(ethylene glycol) was used (not shown) but this treatment was very toxic to the cells.

The liposomes contained a dimannosyldiacylglycerol extracted from *Micrococcus lysodeikticus* [14], which has been reported to bind to concanavalin A [15]. However, binding of vesicles to the cells was probably due to glycoproteins in the lipid vesicles, since the same amount of fluorescence was associated with the cells after addition of varying concentrations of poly(ethylene glycol) to cell-vesicle mixtures where the glycolipid had been omitted in the preparation of lipid vesicles. This indicates that, under the conditions used here, i.e., the use of uncharged lipid vesicles, the association of vesicles to the cells depends on interactions between concanavalin A and glycoproteins [16].

Significant amounts of non-elutable RT-1 antigen were detected on the cells after addition of poly(ethylene glycol) to cells incubated with concanavalin A and lipid vesicles (Table I). The antigen concentration on the cells was approximately 10% of the amount present on a WF lymphocyte. This was calculated from the fluorescence obtained after incubating WF lymphocytes with anti-WF serum and the FITC-labeled antibody under the same conditions as for the modified cells.

The binding of the lectin to the cells and its subsequent elution were investigated both with ^{125}I -labeled concanavalin A and with antibodies against the lectin (Table II). The analysis using

^{125}I -labeled lectin demonstrated the total amount of concanavalin A associated with the cells, while the antibodies selectively detected the lectin exposed on the cell surface. The cells were not incubated with lipid vesicles in these experiments, since they might have sterically hindered the binding of the antibody. Some of the membrane components to which the lectins were bound appear to be shed, since the amount of lectin decreased with prolonged incubation. The decrease was more pronounced when studied by the antibody method. This indicates that some of the lectin molecules were either deeply bound into the cell membrane or capped and internalized. 'Cementing effects' of poly(ethylene glycol) [2] and multi-point attachment of the lectin decrease the amount of lectin that can be eluted after treatment with higher concentrations of poly(ethylene glycol) or after prolonged incubation periods.

The cells were incubated with concanavalin A for 15 min to generate a 'cell-bound receptor' for carbohydrates. During this incubation the concentration of concanavalin A was approx. $5\text{ }\mu\text{g/ml}$ which is close to the optimal concentration for mitogenic stimulation ($8\text{ }\mu\text{g/ml}$) and should have induced mitosis if this process had been allowed to continue.

After dilution of the cell suspension and addition of lipid vesicles, the concentration of the lectin was reduced to about $0.4\text{ }\mu\text{g/ml}$. However, the incubation time was not sufficient to induce cell mitosis. The poly(ethylene glycol) treatment, which induced fusion between cells and lipid vesicles, reduced the concanavalin A-stimulated incorporation of [^3H]thymidine by approx. 50%, as compared to untreated cells. A major part of the reduction could be due to the fact that a proportion of the cells is damaged by the poly(ethylene glycol) treatment. The population of damaged cells includes dead cells (21%) and partially inactivated cells. In addition, the release of lectin-binding glycoproteins from damaged cells would tend to inhibit the mitogenic effect of the lectin [23]. A more speculative explanation would be that the stimulation was affected by the incorporation of glycoproteins and glycolipids in the cell membrane, which has been reported by Jakobovits et al. [8].

It is noteworthy that the cells did become sensi-

tive to lysis by anti-RT-1 serum in the presence of complement. Most of the RT-1 antigen remained detectable on the cells for less than 1 h. The elimination of the WF antigen fits well with the report that, after transferring the receptor for the thyrotropic hormone, the cells could only be stimulated by the hormone during 150 min after fusion [3]. In contrast, a 50 h half-life for some transferred membrane proteins has been reported using radioactively labeled membrane proteins [4]. In the present investigation and also in Ref. 3, the half-life of membrane-incorporated, biologically accessible antigen was in the order of 1–2 h. However, using radioisotope-labeled proteins, internalized proteins are also measured, and thus, these can account for a longer half life.

In conclusion, the described method involving the creation of cell-bound concanavalin A receptors for carbohydrates to optimize attachment of liposomes, and subsequent fusion by poly(ethylene glycol) treatment provides an opportunity to insert purified membrane glycoproteins into liposomes and subsequently introduce them into recipient cells. Such a technique has great potential, since it provides new opportunities of investigating the functions of the cell membrane. Such functions would include induction of proliferative responses, receptor-ligand interactions, cell-cell interactions and signal transfer through the cell membrane. Elimination of the introduced component is an obvious limitation and ways to prolong the half-life must be further investigated.

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